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(71) Applicant *(for all designated States except US)*: DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: YU, Shukun [CN/SE]; Gunnar Hejdemans Gata 29, S-212 40 Malmo (SE). BOJSEN, Kirsten [DK/DK]; Hvidehusvej 13, DK-3450 Allerod (DK). KRAGH, Karsten, Mathias [DK/DK]; Stavtrupvej 139 A, DK-8260 Viby J (DK). BOJKO, Maja [DK/DK]; Frugtparken 11, DK-2820 Gentofte (DK). NIELSEN, John [DK/DK]; Engvej 38, DK-2300 Copenhagen S (DK). MAR-CUSSEN, Jan [DK/DK]; Knabrostraede 25, 2., DK-1210 Copenhagen K (DK). CHRISTENSEN, Tove, Martel, Ida, Elsa [DK/DK]; Søengen 30, DK-3450 Allerød (DK).

(74) Agent: HARDING, Charles, Thomas; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).

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(54) Title: USE OF α-1,4-GLUCAN LYASE FOR PREPARATION OF 1,5-D-ANHYDROFRUCTOSE

(57) Abstract

A method of preparing the sugar 1,5-D-anhydrofructose is described. The method comprises treating an α-1,4-glucan with an α-1,4-glucan lyase wherein the enzyme is used in substantially pure form. In a preferred embodiment, if the glucan contains links other than and in addition to the α-1,4-links, the α-1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.

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USE OF α-1,4-GLUCAN LYASE FOR PREPARATION OF 1,5-D-ANHYDROFRUCTOSE

The present invention relates to the use of an enzyme, in particular α-1,4-glucan lyase ("GL"), to prepare 1,5-D-anhydrofructose ("AF") from substrates based on α-1,4-glucan.

The present invention also relates to the use of a sugar, in particular 1,5-D-anhydrofructose ("AF"), as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

The present invention relates to the use of 1,5-D-anhydrofructose ("AF") as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

FR-A-2617502 and Baute et al in Phytochemistry [1988] vol. 27 No.11 pp3401-3403 report on the production of AF in *Morchella vulgaris* by an apparent enzymatic reaction. The yield of production of AF is quite low. Despite a reference to a possible enzymatic reaction, neither of these two documents presents any amino acid sequence data for any enzyme let alone any nucleotide sequence information. These documents say that AF can be a precursor for the preparation of the antibiotic pyrone microthecin.

Yu et al in Biochimica et Biophysica Acta [1993] vol 1156 pp313-320 report on the preparation of GL from red seaweed and its use to degrade α-1,4-glucan to produce AF. The yield of production of AF is quite low. Despite a reference to the enzyme GL this document does not present any amino acid sequence data for that enzyme let alone any nucleotide sequence information coding for the same. This document also suggests that the source of GL is just algal.

A typical α-1,4-glucan based substrate is starch. Today, starches have found wide uses in industry mainly because they are cheap raw materials.

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Starch degrading enzymes can be grouped into various categories. The starch hydrolases produce glucose or glucose-oligomers. A second group of starch degrading enzymes are phosphorylases that produce glucose-1-phosphate from starch in the presence of inorganic phosphate.

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AF has also been chemically synthesised - see the work of Lichtenthaler in Tetrahedron Letters Vol 21 pp 1429-1432. However, this chemical synthesis involves a large number of steps and does not yield large quantities of AF.

10   The chemical synthetic route for producing AF is therefore very expensive.

There is therefore a need for a process that can prepare AF in a cheap and easy manner and also in a way that enables large quantities of AF to be made.

15   Furthermore, anti-oxidants are typically used to prevent oxygen having any deleterious effect on a substance such as a foodstuff. Two commonly used anti-oxidants are GRINDOX 142 and GRINDOX 1029. These anti-oxidants contain many components and are quite expensive to make.

20   There is therefore a need to have a simpler and cheaper form of anti-oxidant.

Furthermore, sweeteners are often used in the preparation of foodstuffs and beverages. However, many sweeteners are expensive and complex to prepare.

25   There is therefore a need to have a simpler and cheaper form of sweetener.

According to the present invention there is provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α-1,4-glucan with the enzyme α-1,4-glucan lyase characterised in that enzyme is used in substantially pure form.

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Preferably if the glucan contains links other than and in addition to the α-1,4- links the α-1,4-glucan lyase is used in conjunction with a suitable reagent that can break

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the other links - such as a hydrolase - preferably glucanohydrolase.

Preferably the glucan is starch or a starch fraction prepared chemically or enzymatically. If prepared enzymatically the reaction can be performed before the addition of the α-1,4-glucan lyase or the reactions can be performed simultaneously. The suitable reagent can be an auxiliary enzyme. Preferred auxiliary enzymes are alpha- or beta-amylases. Preferably a debranching enzyme is used. More preferably the auxiliary enzyme is at least one of pullanase or isoamylase.

Preferably the α-1,4-glucan lyase either is bound to a support or, more preferably, is in a dissolved form.

Preferably the enzyme is isolated from either a fungus, preferably *Morchella costata or Morchella vulgaris*, or from a fungally infected algae, preferably *Gracilariopsis lemaneiformis*, or from algae lone, preferably *Gracilariopsis lemaneiformis*.

Preferably the enzyme is isolated and/or further purified from the fungus or from the fungally infected algae or algae alone using a gel that is not degraded by the enzyme.

Preferably the gel is based on dextrin or derivatives thereof.

Preferably the gel is a cyclodextrin - more preferably beta-cyclodextrin.

Preferably the enzyme comprises the amino acid sequence SEQ. ID. No. 1. or the amino acid sequence SEQ. ID. No. 2 or the amino acid sequence SEQ. ID. No. 5 or the amino acid SEQ. I.D. No. 6, or any variant thereof.

In an alternative preferable embodiment, the enzyme comprises any one of the amino acid sequences shown in SEQ. ID. No.s 9 - 11, or any variant thereof.

The term "any variant thereof" means any substitution of, variation of, modification of, replacement of, deletion of or addition of an amino acid from or to the sequence

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providing the resultant enzyme has lyase activity.

Preferably the enzyme is used in combination with amylopectin or dextrin.

5   Preferably, the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.

Preferably the nucleotide sequence is a DNA sequence.

10   Preferably the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4 or SEQ. ID. No. 7 or SEQ. ID. No. 8.

15   In an alternative preferable embodiment, the DNA sequence comprises any one of the sequences that are the same as, or are complementary to, or have substantial homology with, or contain any suitable codon substitutions as shown as SEQ. ID. No.s 12 - 14.

20   The expression "substantial homology" covers homology with respect to structure and/or nucleotide components and/or biological activity.

The expression "contains any suitable codon substitutions" covers any codon replacement or substitution with another codon coding for the same amino acid or any
25   addition or removal thereof providing the resultant enzyme has lyase activity.

In other words, the present invention also covers a modified DNA sequence in which at least one nucleotide has been deleted, substituted or modified or in which at least one additional nucleotide has been inserted so as to encode a polypeptide having the
30   activity of a glucan lyase, preferably having an increased lyase activity.

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Preferably the starch is used in high concentration - such as up to about 25% solution.

Preferably the substrate is treated with the enzyme in the presence of a buffer.

More preferably the substrate is treated with the enzyme in the presence of substantially pure water.

Preferably the substrate is treated with the enzyme in the absence of a co-factor.

According to the present invention there is also provided a method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α-1,4-glucan with the enzyme α-1,4-glucan lyase characterised in that enzyme comprises the amino acid sequence SEQ. ID. No. 1. or the amino acid sequence SEQ. ID. No. 2 or the amino acid sequence SEQ. ID. No. 5. or the amino acid sequence SEQ. ID. No. 6, or any one of the amino acid sequences SEQ. I.D. No.s 9-11, or any variant thereof.

According to the present invention there is also provided the sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.

AF prepared by the present method was confirmed and characterised by ^{13}C NMR.

One of key advantages of the present method is that the sugar 1,5-D-anhydrofructose can be prepared in much larger quantities than before and by a method that is relatively easier and cheaper than the known processes. For example the sugar can now be prepared in amounts of for example greater than 100g - such as 500g - compared to the prior art methods when only much smaller amounts were and could be produced - such as micro gram amounts.

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Typical reactions that can be catalyzed by GL can be summarised as follows:

1). Amylopectin --------------------------→ AF + limit dextrin

5   2). Amylose ----------------------------→ AF + limit dextrin

3). Dextrin ----------------------------→ AF + glucose

In reaction 1), the ratio of the two products depend on the structure of amylopectin
10   or the distribution of α-1,6-glucosidic linkages in the amylopectin molecules.

In reaction 2) and 3), the ratio of the products depends on the degree of
polymerisation (DP) number of the substrate. In reaction 3 the ratio between AF and
glucose depends upon the DP. For example if the dextrin contains 10 glucose units
15   the ratio AF:glucose would be 9:1.

Another advantage of the present invention is that glucans that contain links other
than α-1,4- links can be substantially degraded - whereas before only partial
degradation was achieved. The substantial degradation of the 1,5-D-anhydrofructose
20   precursor is one of the factors leading to the increased yields of 1,5-D-
anhydrofructose.

Other advantages are AF is a naturally occurring substance and therefore it has a
potential for human purposes. For example, it can be converted to the antibiotic
25   microthecin by AF dehydrase. Antibiotics are known for their uses in food bio-
preservation, which is an important area in food technology. However, to date, the
preparation of AF and also microthecin has had a number of disadvantages. For
example, only small quantities could be produced. Also, the process was costly.

30   The present invention overcomes these problems by providing a larger production of
and much cheaper production of AF and so also other products such as microthecin.
In this regard, it is possible to prepare gram to kilogram amounts of AF.

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A further advanatge is that the lyase is stable for at least one year at 4°C and can be lyophilized without loss of activity.

Another advantage is that the lyase produces AF directly from starches and does not need the presence of any co-factors.

Another advantage is that the enzyme can be used in pure water. This result is very surprising.

Based on the simple properties of the present lyase, one can expect that the production cost of AF will be comparable to that of glucose. This is especially advantageous that the present lyase does not necessarily require the presence of any co-factors which are generally very expensive.

In general α-1,4-glucans can be used as substrate for the enzyme.

As a preferred substrate, starch is used.

In a preferred process, soluble or gelatinized starch or starch hydrolysate are used. The starch hydrolysates can be prepared either chemically or enzymatically.

If an enzyme is used for the partial starch degradation the enzyme can either be added before the addition of the lyase or any other additional starch degrading reagent (such as the enzyme glucanohydrolase) which may be added simultaneously.

The lyase will convert the glucan to AF. The enzyme will attach the substrate from the non reducing end and leave only the reducing sugar unconverted. The residual glucose can be removed by known methods some of which have been described here.

Using the reaction described here pure AF can be produced and also in large amounts.

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In one embodiment, the α-1,4-glucan lyase is purified from the fungally infected algae - such as *Gracilariopsis lemaneiformis* - by affinity chromatography on β-cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α-1,4-glucans.

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The fungal lyase isolated from fungal infected *Gracilariopsis lemaneiformis* is characterized as having a pH optimum at 3.5-7.5 when amylopectin is used, a temperature optimum at 50°C and a pI of 3.9.

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In another embodiment, the α-1,4-glucan lyase is purified from the fungus *Morchella costata* by affinity chromatography on β-cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α-1,4-glucans.

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The fungal lyase shows a pI around 5.4 as determined by isoelectric focusing on gels with pH gradient of 3 to 9. The molecular weight determined by SDS-PAGE on 8-25% gradient gels was 110 kDa. The enzyme exhibited a pH optimum in the range pH 5-7. The temperature optimum was found to be between 30-45 °C.

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In another embodiment, the α-1,4-glucan lyase is purified from the fungus *Morchella vulgaris* by affinity chromatography on β-cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α-1,4-glucans.

25

In another embodiment, the α-1,4-glucan lyase is purified from algae - such as *Gracilariopsis lemaneiformis* - by affinity chromatography on β-cyclodextrin Sepharose, ion exchange chromatography on Mono Q HR 5/5 and gel filtration on Superose 12 columns. The purified enzyme produces 1,5-anhydro-D-fructose from α-1,4-glucans.

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Typical pH and temperature optima for the lyase catalyzed reaction for some of the GL enzymes according to the present invention are as follows:

GL sources	Optimal pH	Optimal pH range	Optimal temperature
M. costata	6.5	5.5-7.5	37 C; 40 C*
M. vulgaris	6.4	5.9-7.6	43 C; 48 C*
Fungal infected *Gracilariopsis*			
lemaneiformis	3.8	3.7-4.1	40 C; 45 C*

*Parameters determined using glycogen as substrate; other parameters determined using amylopectin as substrate.

The enzymes of the present invention convert amylose and amylopectin to 1,5-anhydrofructose.

Among the maltosaccharides tested, we found that the lyase showed low activity towards maltose, and lower activity to maltotriose and maltoheptaose with the highest activity to maltotetraose and maltopentaose. The enzyme showed no substrate inhibition up to a concentration 10 mg ml^{-1} among these maltosaccharides.

The enzymes from each of the preferred sources has been sequenced and the amino acid sequences are presented later. Also presented later are the DNA sequences coding for the enzymes.

The present invention therefore describes a new starch degrading enzyme - namely a new α-1,4-glucan lyase. This is an enzyme that has been purified and characterized for the first time.

10

As mentioned above, the present invention also relates to some specific uses of AF.

In particular, the present invention relates to the use of 1,5-D-anhydrofructose ("AF"), as an anti-oxidant, in particular as an anti-oxidant for food stuffs and beverages.

Therefore according to the present invention there is provided the use of 1,5-D-anhydrofructose (AF) as an anti-oxidant.

Preferably AF is or is used in an edible substance.

Preferably AF is used in or as a foodstuff or beverage.

Preferably, AF is used in combination with another anti-oxidant.

Preferably the AF is prepared by the method according to the present invention.

The main advantages of using AF as an anti-oxidant are that it is a natural product, it is non-metabolisable, it is easy to manufacture, it is water-soluble, and it is generally non-toxic.

In a preferred embodiment the present invention therefore relates to the enzymatic preparation of pure AF which can be used as an attractive water soluble antioxidant for food and non-food purposes. In the application examples are given for the use of AF as an antioxidant in food formulations.

In the accompanying examples it is seen that AF is comparable with known high quality commercial available food antioxidants.

Non-food examples include use in polymer chemistry as oxygen scavengers during the synthesis of polymers. Also, AF could be used for the synthesis of bio-degradable plastic.

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Experiments have shown that AF can be an efficient reducing agent (antioxidant), as it can easily reduce 3,5-dinitrosalicylic acid to 3-amino-5-nitrosalicylic acid.

AF is a naturally occurring substance and therefore it has a tremendous potential for use as an acceptable antioxidant. AF can also be converted into the antibiotic microthecin by AF dehydrase. Antibiotics are known for their uses in food biopreservation, an important area in food biotechnology.

In another aspect, the present invention also relates to the use of 1,5-D-anhydrofructose as a sweetener, in particular as a sweetener for foodstuffs and beverages, preferably human foodstuffs and beverages.

Thus according to this aspect of the present invention there is provided the use of 1,5-D-anhydrofructose as a sweetener.

Preferably the AF is used as or in a human foodstuff or beverage.

The AF may be used in any desired amount such as a 5% soution or 100mg/kg to 500 mg/kg.

The advantages of using AF as a sweetener are that it is a natural product, it is generally non-toxic, it is water soluble, it is non-metabolisable and it is easy to manufacture.

The present invention therefore also relates to a novel application of AF as a sweetener.

Preferably the AF is prepared by the method according to the present invention.

Further aspects of the present invention include:

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a method of preparing the enzyme α-1,4-glucan lyase (GL) comprising isolating the enzyme from a fungally infected algae, fungus or algae alone;

an enzyme comprising the amino acid sequence SEQ. ID. No. 1. or SEQ. ID.

5   No. 2 or SEQ. ID. No. 5. or SEQ. ID. No. 6, or any variant thereof;

an enzyme comprising the amino acid sequence SEQ. ID. No. 9. or SEQ. ID. No. 10 or SEQ. ID. No. 11, or any variant thereof;

10   a nucleotide sequence coding for the enzyme α-1,4-glucan lyase, preferably wherein the sequence is not in its natural enviroment (i.e. it does not form part of the natural genome of a cellular organism capable of expressing the enzyme, preferably wherein the nucleotide sequence is a DNA sequence;

15   a nucleotide sequence wherein the DNA sequence comprises at least a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4 or SEQ. ID. No. 7 or SEQ. ID. No. 8, preferably wherein the sequence is in isolated form;

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a nucleotide sequence wherein the DNA sequence comprises at least a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 12 or SEQ. ID. No. 13 or SEQ. ID. No. 14, preferably wherein the sequence is in

25   isolated form; and

the use of beta-cyclodextrin to purify an enzyme, preferably GL.

Other preferred embodiments of the present invention include any one of the

30   following: A transformed host organism having the capability of producing AF as a consequence of the introduction of a DNA sequence as herein described; such a transformed host organism which is a microorganism - preferably wherein the host

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organism is selected from the group consisting of bacteria, moulds, fungi and yeast; preferably the host organism is selected from the group consisting of *Saccharomyces, Kluyveromyces, Aspergillus, Trichoderma Hansenula, Pichia, Bacillus Streptomyces, Eschericia* such as *Aspergillus oryzae, Saccharomyces cerevisiae, bacillus sublilis,*

5   *Bacillus amyloliquefascien, Eschericia coli.*; A method for preparing the sugar 1,5-D-anhydrofructose comprising the use of a transformed host organism expressing a nucleotide sequence encoding the enzyme α-1,4-glucan lyase, preferably wherein the nucleotide sequence is a DNA sequence, preferably wherein the DNA sequence is one of the sequences hereinbefore described; A vector incorporating a nucleotide sequence

10   as hereinbefore described, preferably wherein the vector is a replication vector, preferably wherein the vector is an expression vector containing the nucleotide sequence downstream from a promoter sequence, preferably the vector includes a marker (such as a resistance marker); Cellular organisms, or cell line, transformed with such a vector;   A method of producing the product α-1,4-glucan lyase or any

15   nucleotide sequence or part thereof coding for same, which comprises culturing such an organism (or cells from a cell line) transfected with such a vector and recovering the product.

In particular, in the expression systems, the enzyme should preferably be secreted to

20   ease its purification. To do so the DNA encoding the mature enzyme is fused to a -signal sequence, a promoter and a terminator from the chosen host.

For expression in *Aspergillus niger* the gpdA (from the Glyceraldehyde-3-phosphate dehydrogenase gene of *Aspergillus nidulans*) promoter and signal sequence is fused

25   to the 5' end of the DNA encoding the mature lyase. The terminator sequence from the *A. niger* trpC gene is placed 3' to the gene (Punt, P.J. et al 1991 - (1991): J. Biotech. 17, 19-34). This construction is inserted into a vector containing a replication origin and selection origin for *E. coli* and a selection marker for *A. niger*. Examples of selection markers for *A. niger* are the amdS gene, the argB gene, the

30   pyrG gene, the hygB gene, the BmlR gene which all have been used for selection of transformants. This plasmid can be transformed into *A. niger* and the mature lyase can be recovered from the culture medium of the transformants. Eventually the

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construction could be transformed into a protease deficient strain to reduce the proteolytic degradation of the lyase in the culture medium (Archer D.B. et al 1992 - Biotechnol. Lett. 14, 357-362).

5   Instead of *Aspergillus niger* as host, other industrial important microorganisms for which good expression systems are known could be used such as: *Aspergillus oryzae, Aspergillus sp., Trichoderma sp., Saccharomyces cerevisiae, Kluyveromyces sp., Hansenula sp., Pichia sp., Bacillus subtilis, B. amyloliquefaciens, Bacillus sp., Streptomyces sp.* or *E. coli.*

10

The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 20 June 1994:

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E.Coli containing plasmid pGL1 (NCIMB 40652) - [ref. DH5alpha-pGL1]; and

E.Coli containing plasmid pGL2 (NCIMB 40653) - [ref. DH5alpha-pGL2].

20   The following sample was accepted as a deposit in accordance with the Budapest Treaty at the recognised depositary The Culture Collection of Algae and Protozoa (CCAP) at Dunstaffnage Marine Laboratory PO Box 3, Oban, Argyll, Scotland, United Kingdom, PA34 4AD on 11 October 1994:

25   Fungally infected *Gracilariopsis lemaneiformis* (CCAP 1373/1) - [ref. GLQ-1 (Qingdao)].

Thus highly preferred embodiments of the present invention include a GL enzyme obtainable from the expression of the GL coding sequences present in plasmids that
30   are the subject of either deposit NCIMB 40652 or deposit NCIMB 40653; and a GL enzyme obtainable from the fungally infected algae that is the subject of deposit CCAP 1373/1.

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The following samples were deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 3 October 1994:

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E. Coli containing plasmid pMC (NCIMB 40687) - [ref. DH5alpha-pMC];

E. Coli containing plasmid pMV1 (NCIMB 40688) - [ref. DH5alpha-pMV1]; and.

10   *E. Coli* containing plasmid pMV2 (NCIMB 40689) - [ref. DH5alpha-pMV2].

Plasmid pMC is a pBluescript II KS containing a 4.1 kb fragment isolated from a genomic library constructed from *Morchella costata*. The fragment contains a gene coding for α-1,4-glucan lyase.

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Plasmid pMV1 is a pBluescript II KS containing a 2.45 kb fragment isolated from a genomic library constructed from *Morchella vulgaris*. The fragment contains the 5' end of a gene coding for α-1,4-glucan lyase.

20   Plasmid MV2 is a pPUC19 containing a 3.1 kb fragment isolated from a genomic library constructed from *Morchella vulgaris*. The fragment contains the 3' end of a gene coding for α-1,4-glucan lyase.

In the following discussions, MC represents *Morchella costata* and MV represents
25   *Morchella vulgaris*.

As mentioned, the GL coding sequence from *Morchella vulgaris* was contained in two plasmids. With reference to Figure 15 pMV1 contains the nucleotides from position 454 to position 2902; and pMV2 contains the nucleotides downstream from (and
30   including) position 2897. With reference to Figures 12 and 13, to ligate the coding sequences one can digest pMV2 with restriction enzymes EcoRI and BamHI and then insert the relevant fragment into pMV1 digested with restriction enzymes EcoRI and

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BamHI.

Thus highly preferred embodiments of the present invention include a GL enzyme obtainable from the expression of the GL coding sequences present in plasmids that are the subject of either deposit NCIMB 40687 or deposit NCIMB 40688 and deposit NCIMB 40689.

The following sample was also accepted as a deposit in accordance with the Budapest Treaty at the recognised depositary The Culture Collection of Algae and Protozoa (CCAP) at Dunstaffnage Marine Laboratory PO Box 3, Oban, Argyll, Scotland, United Kingdom, PA34 4AD on 11 October 1994:

Fungally infected *Gracilariopsis lemaneiformis* (CCAP 1373/2) - [ref. GLSC-1 (California)].

Thus a highly preferred embodiment of the present invention includes a GL enzyme obtainable from the algae that is the subject of deposit CCAP 1373/2.

The present invention will now be described only by way of example.

In the following Examples reference is made to the accompanying figures in which:

Figure 1 shows stained fungally infected algae;

Figure 2 shows stained fungally infected algae;

Figure 3 shows sections of fungal hypha;

Figure 4 shows sections of fungally infected algae;

Figure 5 shows a section of fungally infected algae;

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Figure 6 shows a plasmid map of pGL1;

Figure 7 shows a plasmid map of pGL2;

5   Figure 8 shows the amino acid sequence represented as SEQ. I.D. No.3 showing
positions of the peptide fragments that were sequenced;

Figure 9 shows the alignment of SEQ. I.D. No. 1 with SEQ. I.D. No.2;

10   Figure 10 is a microphotograph;

Figure 11 shows a plasmid map of pMC;

Figure 12 shows a plasmid map of pMV1;
15
Figure 13 shows a plasmid map of pMV2;

Figure 14 shows the GL coding sequence and part of the 5' and 3' non-translated
regions for genomic DNA obtained from *Morchella costata*;
20
Figure 15 shows the GL coding sequence and part of the 5' and 3' non-translated
regions for genomic DNA obtained from *Morchella vulgaris*;

Figure 16 shows a comparison of the GL coding sequences and non-translated regions
25   from *Morchella costata* and *Morchella vulgaris*;

Figure 17 shows the amino acid sequence represented as SEQ. I.D. No. 5 showing
positions of the peptide fragments that were sequenced;

30   Figure 18 shows the amino acid sequence represented as SEQ. I.D. No. 6 showing
positions of the peptide fragments that were sequenced;

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Figure 19 shows a graph of oxygen consumption with and without the presence of AF; and

Figure 20 shows a TLC plate.

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In more detail, Figure 1 shows Calcoflour White stainings revealing fungi in upper part and lower part of *Gracilariopsis lemaneiformis* (108x and 294x).

Figure 2 shows PAS/Anilinblue Black staining of *Gracilariopsis lemaneiformis* with

10   fungi. The fungi have a significant higher content of carbohydrates.

Figure 3 shows a micrograph showing longitudinal and grazing sections of two thin-walled fungal hypha (f) growing between thick walls (w) of algal cells. Note thylacoid membranes in the algal chloroplast (arrows).

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Figure 4 shows the antisense detections with clone 2 probe (upper row) appear to be restricted to the fungi illustrated by Calcoflour White staining of the succeeding section (lower row) (46x and 108x).

20   Figure 5 shows intense antisense detections with clone 2 probe are found over the fungi in *Gracilariopsis lemaneiformis* (294x).

Figure 6 shows a map of plasmid pGL1 - which is a pBluescript II KS containing a 3.8 kb fragment isolated from a genomic library constructed from fungal infected

25   *Gracilariopsis lemaneiformis*. The fragment contains a gene coding for alpha-1,4-glucan lyase.

Figure 7 shows a map of plasmid pGL2 - which is a pBluescript II SK containing a 3.6 kb fragment isolated from a genomic library constructed from fungal infected

30   *Gracilariopsis lemaneiformis*. The fragment contains a gene coding for alpha-1,4-glucan lyase.

Figure 9 shows the alignment of SEQ. I.D. No. 1 (GL1) with SEQ. I.D. No.2 (GL2). The total number of residues for GL1 is 1088; and the total number of residues for GL2 is 1091. In making the comparison, a structure-genetic matrix was used (Open gap cost: 10; Unit gap cost: 2). In Figure 9 the character to show that two aligned residues are identical is ':'; and the character to show that two aligned residues are similar is '.'. Amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. Overall there is an identity of 845 amino acids (i.e. 77.67%); a similarity of 60 amino acids (5.51%). The number of gaps inserted in GL1 are 3 and the number of gaps inserted in GL2 are 2.

Figure 10 is a microphotograph of a fungal hypha (f) growing between the algal walls (w). Note grains of floridean starch (s) and thylakoids (arrows) in the algal cell.

In Figure 14, the total number of bases is 4726 - and the DNA sequence composition is: 1336 A; 1070 C; 1051 G; 1269 T. The ATG start codon is shown in bold. The introns are underlined. The stop codon is shown in italics.

In Figure 15, the total number of bases is 4670 - and the DNA sequence composition is: 1253 A; 1072 C; 1080 G; 1265 T. The ATG start codon is shown in bold. The introns are underlined. The stop codon is shown in italics.

In Figure 16, the two aligned sequences are those obtained from MC (total number of residues: 1066) and MV (total number of residues: 1070). The comparison matrix used was a structure-genetic matrix (Open gap cost: 10; Unit gap cost : 2). In this Figure, the character to show that two aligned residues are identical is ':'. The character to show that two aligned residues are similar is '.'. The amino acids said to be 'similar' are: A,S,T; D,E; N,Q; R,K; I,L,M,V; F,Y,W. Overall there is: Identity: 920 (86.30%); Similarity: 51 (4.78%). The number of gaps inserted in MC is 1 and the number of gaps inserted in MV is 1.

20

In the attached sequence listings: SEQ. I.D.No. 5 is the amino-acid sequence for GL obtained from *Morchella costata*; SEQ. I.D.No. 6 is the amino-acid sequence for GL obtained from *Morchella vulgaris*; SEQ. I.D. No. 7 is the nucleotide coding sequence for GL obtained from *Morchella costata;* and SEQ. I.D.No. 8 is the nucleotide coding sequence for GL obtained from *Morchella vulgaris*.

In SEQ. I.D. No. 5 the total number of residues is 1066. The GL enzyme has an amino acid composition of:

46 Ala	13 Cys	25 His	18 Met	73 Thr
50 Arg	37 Gln	54 Ile	43 Phe	23 Trp
56 Asn	55 Glu	70 Leu	56 Pro	71 Tyr
75 Asp	89 Gly	71 Lys	63 Ser	78 Val

In SEQ.I.D. No. 6 the total number of residues is 1070. The GL enzyme has an amino acid composition of:

51 Ala	13 Cys	22 His	17 Met	71 Thr
50 Arg	40 Gln	57 Ile	45 Phe	24 Trp
62 Asn	58 Glu	74 Leu	62 Pro	69 Tyr
74 Asp	87 Gly	61 Lys	55 Ser	78 Val

EXPERIMENTS

1   THE SOLUBLE ENZYME SYSTEM:

1.1. Effect of pH on the stability and activity of the lyase isolated from fugal infected *Gracilariopsis lemaneiformis*.

Two buffer systems, namely HOAc and NaOAc and sodium citrate - citric acid in a concentration of 5 mM - were tested at 37°C. The pH range tested was from pH 3 to pH 5.2. The lyase showed maximum activity in a pH range between 3.6 to 4.2. At

21

pH 3, the stability and activity of the enzyme decreased by about 90%. At pH 5.2, the activity decreased by about 64%. However, the enzyme was considerably more stable at this pH than at pH 3, as the AF yield obtained at pH 5.2 was 75% of the AF yield obtained at pH 3.8. Slightly higher AF yield was obtained in the HOAc and NaOAc buffer than in citrate buffer. This is not due to any differential effect of the two buffers (final conc. is 125 μM in the AF assay mixture) in the AF assay method.

1.2. Effect of temperature on the activity and stability of the lyase.

This experiment was conducted at optimal pH range. At 25°C the production of AF was linear up to at least 9 days. This indicates that no loss of activity and stability of the lyase occurred within 9 days. With increasing temperature, the stability of the enzyme decreased.

The half life of the enzyme activity at the following temperature was:

30°C   5 days
37°C   2.5 days
40°C   less than 1 day
50°C   less than 1 day

1.3. Effect of substrate concentration on the stability of the lyase and AF yield.

It was observed that amylopectin and dextrins have a stabilizing effect on the lyase while the smallest substrate maltose does not. This was verified for both the soluble enzyme system and the immobilized enzyme system.

AF yield increases with the increase in amylopectin concentration up to 25%. In the case of dextrin, the AF yield decreases when the concentration exceeds 30% (30%, 40% and 50% were tested).

22

1.4 Activation and inactivation of lyase

No metal ions are found necessary for the activity and the enzyme catalysed reaction
can surprisingly proceed in pure water. The fact that the addition of EDTA in the
5   reaction mixture up to 20 mM had little effect on the activity clearly demonstrates
that metal ions are not essential for the activity of the lyase enzyme according to the
present invention.

This means that in the AF purification step, the ion exchange chromatography step
10   that takes away salts from the reaction system can be omitted, if water is used as
reaction medium. However, inclusion of NaCl in the reaction mixture in a
concentration of 0.85% (0.145 M) can increase the AF yield up to 1-fold.

1.5. Substrate Specificity

15

Upon cooling solubilized starch will tend to form rigid gels when the starch
concentration becomes to high. Therefore it is an advantage to utilize partly degraded
starch as substrate for the 1,4-glucan lyase.

20   The specificity of α-1,4-glucan lyase isolated from M. costata for different
oligosaccharides was tested. The oligosaccharides were maltose (G2), maltotriose
(G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6) and maltoheptaose
(G7). The oligosaccharides were dissolved in H_2O at a concentration of 8 mg/ml. The
enzyme assay contained 150 μl substrate G2/G3/G4/G5/G6/G7, 120 μl 0.1M MES
25   pH 6.3 and 30 μl purified enzyme. The reaction mixture was incubated for 60 min
at 30°C. Afterwards the reaction was stopped by boiling for 3 min and 900 μl
absolute ethanol was added for precipitation. After centrifugation at 20.000 x g for
5 min at 4°C the supernatant was transferred to a new eppendorf tube and lyophilized.

30   The freeze-dried samples were dissolved in 1000 μl H_2O and were filtrated through
a 0.22 μm Millipore filter before 25 μl of the sample was loaded on the Dionex
HPLC.

23

1.7 **HPLC**

Analytical procedures.

Analyses were performed on a Dionex 4500i chromatography system consisting of a
GPM-2 pump and a PED detector which was used in pulse-amperometric detection
mode.

The anion exchange columns were a CarboPac PA-100 (4 x 250 mm) and a CarboPac
PA-100 guard column (3 x 25 mm) from Dionex.

The eluent were 200 mM sodium hydroxide (A), 500 mM sodium acetate (B) and 18
M ohm de-ionized water (C) . The pump was programmed in 2 different ways,
method no. 1 and method no. 2:

Method no. 1:

Time, min	0.0	3.0	3.1	26.9	29.0
% A	10	10	50	50	10
% B	0	0	0	32	0
% C	90	90	50	18	90

Method no. 2:

Time, min.	0.0	30
% A	10	10
% B	0	0
% C	90	90

24

Standards:

Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose and
maltoheptaose (all from Sigma) and 1,5-anhydrofructose were used as standards. All
5   compounds were dissolved in 18 M ohm de-ionized water which was filtered through
a 0.22 μm Millipore filter before use.

1.7 Results:

10   The analyses show that the purified enzyme which was isolated from M. costata
indeed was able to use maltooligosaccharides as substrate 1 for 1,5-anhydrofructose
formation.

When maltose was used as substrate, almost no 1,5-anhydrofructose was formed but
15   when the other maltooligosaccharides (G3-G7) were used, high amounts of this
compound were produced.

It is clear that higher amounts of 1,5-anhydrofructose were obtained when a longer
maltooligosaccharide was used.

20

This observation corresponds perfectly well with the theory of the lyase forming 1,5-
anhydrofructose from the non-reducing end of the substrate, leaving only the terminal
glucose molecule unchanged.

25   1.8 Formation of AF

α-1,4-glucan lyase from M.costata hydrolyses starch to the end-product 1,5-
anhydrofructose. The end-product was shown by HPLC, method 2. The enzyme assay
contained 500 μl amylopectin (20 mg/ml, dissolved in H_2O), 400 μl 0.1 M MES pH
30   6.3 and 100 μl purified enzyme. The reaction mixture was incubated at 30°C and the
reaction was stopped by boiling after 30 or 120 min incubation. High-molecular
oligosaccharides were precipitated by addition of 3 vol abs. ethanol and the sample

25

was centrifuged and freeze-dried as described above. The samples were dissolved in
125 μl H_2O and 25 μl were applied on the HPLC column.

The HPLC elution profile clearly shows that α-1,4-glucan lyase from M.costata
5   produces 1,5-anhydrofructose by hydrolysis of starch. Equal amounts of 1,5-
anhydrofructose were found after 30 and 120 min. incubation which indicate that the
enzyme activity is not inhibited by the endproduct 1,5-anhydrofructose.

^{13}C NMR spectra (water) of AF prepared in this way shows that it adopts one major
10 form giving rise to the following signals: δ 93.5 (quart, C-2), 81.5 (CH, C-5), 77.7
(CH, C-3), 72.6 (CH_2, C-1), 69,8 (CH, C-4), 62.0 (CH_2, C-6). Assignments are
based on H-H C-H and C-H 2D correlation spectra.

1.6. The cooperative effect of lyase with pullulanase and isoamylase.

15

As it can be seen from Table 1, the inclusion of pullulanase in the reaction mixture
will obviously increase the AF yield by about 15-23%, depending on whether soluble
starch or amylopectin is used as substrate.

26

Table The cooperation of pullulanase and lyase in the production of AF.

Substrate	Lyase	Pullulanase	AF Yield (%)	Glc Yield (%)
Solubl. Starch	+	-	51	0
	-	+	0	0.37
	+	+	66.0	3.9
Amylo -pectin	+	-	48.0	0
	-	+	0	0.33
	+	+	71.3	3.7

+, enzyme added, - enzyme omitted.

The reaction mixture contained 0.3 ml 2% potato amylopectin (Sigma) in water or 0.3 ml 2% soluble starch (Merck), 2 μl lyase and 0.36 units pullulanase (BM) as indicated.

5

The reaction was carried out at 30°C for 1 day. At the end of the reaction, samples were taken for AF and Glc analysis.

In the case of isoamylase, the advantage is that the optimal pH of the lyase overlaps

10   with that of Pseudomonas isoamylase (pH 3.0-4.5). The problem, however, is that isoamylase will produce an excess amount of long chain amylose that precipitates from the solution, and therefore is no longer suitable as a substrate for the lyase. It can be expected that the cooperation of the lyase with isoamylase will be efficient, if the chain of amylose is not too long.

27

2.   THE IMMOBILIZED ENZYME SYSTEM

Immobilization of the lyase was achieved by using succinimide-activated Sepharose
(Affigel 15 gel, Bio-Rad) and glutaradehye-activated Silica gel (BM). The recovery
of lyase activity after immobilization on Affigel 15 gel was between 40% to 50%.
There may be some lyase that is still active after immobilization, but is inaccessible
to the substrate because of the steric hindrance, especially in the case of
macromolecules like starches. Immobilized enzymes used in the industry usually have
an activity recovery of around 50%.

The most interesting thing of the Affigel 15 gel immobilized lyase is that its stability
has been greatly improved at pH 5.5. When the column was operated at this pH, the
stability was at least 16 days long. The pH shift in the stability is very important
considering the optimal pH of pullulanase which is around pH 5.5. This is the
prerequisite for the lyase and pullulanase to cooperate efficiently in the same reactor
with the same physico-chemical environment. The soluble lyase has an optimal pH
between 3.6 and 4.2, and at this pH range pullulanase shows little or no activity.

With the silica gel immobilized lyase, the activity recovery is very high, around 80-
100%. However, the silica gel immobilized enzyme was not stable when the column
was operated neither at pH 3.8 nor pH 5.5. It is possible that some lyase was ad-
sorbed on the surface of the silica gel beads and was slowly released from the silica
gel after each washing of the column. It may therefore be the adsorbed lyase that
contributes to the high recovery rate and the decrease in column activity.

28

3.   PURIFICATION OF AF

3.1. The lyase-Amylopectin/Soluble Starch System

In this system, the reaction system contained AF, limit dextrin, the lyase, and buffer salts at the end of the reaction. AF was separated from the macromolecules (limit dextrin and the lyase) by ethanol (final conc. 50%) precipitation. Unprecipitated low-molecular-weight amylopectin was separated by ultrafiltration using Amicon YM3 membranes (cut-off 3,000). Ethanol was removed by evaporation at 40°C in a rotary evaporator. Buffer salts were removed from AF by mixed ion exchangers. Purified solid AF was obtained by freeze-drying.

3.2. The Lyase-Pullulanase/Amylopectin/Soluble Starch System.

In this system the final products are AF and glucose. If at least a substantially pure sample of AF is to be prepared, the by-product glucose must be removed. This can be achieved by enzymatic methods. First the glucose is converted into gluconic acid and hydrogen peroxide by glucose oxidase.

Catalase is needed to dispel H_2O_2 formed. H_2O_2 will oxidize AF into two new compounds which are at present of unknown structure. The other impurities in the AF preparation are the oxidation products of AF. It was observed that AF can slowly be oxidized by air-level of oxygen, especially at high temperature, high AF concentration and long time of exposure.

Gluconic acid was removed together with the buffer salts by ion exchange chromatography.

In this system, the low-molecular-weight amylopectin molecules may alternatively be hydrolysed by amyloglucosidase instead of using ultrafiltration.

29

3.3. The purity checking of AF.

The purity of the AF preparations were confirmed by TLC, Dionex and NMR.

5   3.4 Analysis of the antioxidative activity of anhydro fructose.

Electrochemical oxygen consumption:

Method.

10   The activity of AF was investigated in a methyl linoleate emulsion as described by
     Jorgensen and Skibsted (Z. Lebensm. Unters. Forsch. (1993) 196: 423-429) with
     minor modifications: To 5.00 ml of a 1.33 mM methyl linoleate emulsion in 5.0 mM
     aqueous phosphate buffer with pH = 5.8 and 0.2 w/w % Tween 20 as emulsifier was
     added AF in the following concentrations: 0, 15, 146 and 680 μM. The oxidation in
15   the system was initiated by addition of 50 μl 0.26 M metmyoglobin (MMb) final
     concentration 0.26 mM. Immediately after initiating the reaction the sample was
     injected to a thermostated (25.0 $\pm$ 0.1°C) 70 μl closed cell, effectively excluding
     diffusion of oxygen into the system. The oxygen consumption was measured by a
     Clark electrode, which was connected to a PC data collection program. The relative
20   oxygen concentration (%) was registered every 30s.

Results.

     Curves corresponding to oxygen consumption for the different samples are illustrated
25   in Figure 19. For samples without addition of AF a relative decrease in oxygen
     concentration is seen immediately after injection of the sample. For samples
     containing AF a lag-phase is observed before the curve breaks off and the oxygen
     concentration is reduced. After the lag-phase only a minor reduction in the oxygen
     consumption rate is observed compared to samples without AF added. A tendency for
30   samples having the highest amount of AF to have the longest lag-phase is observed.
     As well the rate for oxygen consumption is lower for these samples, which is seen
     by a smaller slope of the curves compared to the slope for the references (0 μM).

ESR analysis

Method.

Hydroxyl radicals were generated by a Fenton reaction with H_2O_2 (0.17 mM) and $FeSO_4$ (4.8 μM). The generated radicals were trapped by 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 9.7 mM). AF was added in concentrations of 1.3 mM and 6.3 mM. A water soluble extract of rosemary (*Rosmarinus officinalis* L.) was analyzed in a concentration of 0.25 mg/ml (in grams equivalent to 1.26 mM AF). Measurements were carried out at room temperature (20 $\pm$ 1°C) after 120 s and repeated for the same reaction mixture after 300 s with the following spectrometer settings: Center field 3475.60 G; sweep width 55 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; receiver gain 1.00 · 10^5; conversion time 81.92 ms time constant 163.84 ms and sweep time 83.89 s.

Results.

The generated hydroxyl radicals were trapped by DMPO. The spin adduct gives rise to a characteristic 1:2:2:1 ESR spectrum. The peak height of the spectrum is proportional to the quantitative amount of generated spin adduct. Addition of both DMPO and AF will set up a competition between the spin trap and AF. A reduction of peak height will indicate a good scavenging activity of AF.

Table: Peak height of ESR-spectra. H_2O_2 = 0.17mM and Fe^{2+} = 4.8 μM.

Anhydro fructose [mM]	Rosemary extract [mg/ml]	Peak height [120 s]	Peak height [300 s]
0	0	2475	2780
1.3	0	2634	2545
6.3	0	1781	1900

31

At a concentration of 1.3 mM AF no scavenging activity of hydroxyl radicals is seen, at 6.3 mM Af the peak height is reduced, indicating that a part of the generated hydroxyl radicals is scavenged by AF.

5   4.   USE OF AF AS AN ANTI-OXIDANT

EXAMPLE 4.1

Use of AF as an anti-oxidant in a 50% mayonnaise.

10

50% mayonnaise is used for salads, open sandwiches, etc. in both the catering and the retail trades. The low oil content of 50% mayonnaise makes it suitable for low-calorie applications.

15   A typical mayonnaise composition is as follows:

Soya oil	50.0%
Tarragon vinegar (10%)	4.0%
Egg yolk	3.5%
Sugar	3.0%
Salt	1.0%
Potassium sorbate	0.1%
Water	35.2%
MAYODAN 602	3.0%
Lemon flavouring 10251	0.2%

MAYODAN 602 ensures a fine, stable oil dispersion and the required viscosity, thereby providing 50% mayonnaise with a long shelf life.

30   Flavouring 10251 is a natural lemon flavouring which provides mayonnaise with the fresh taste of lemon.

32

Typically the mayonnaise is prepared by the following method:

1) Dry mix the MAYODAN 602, sugar and salt. Disperse in oil in a ratio of 1 part powder to 2 parts oil.

2) Add flavouring and potassium sorbate to the water and pour into the Koruma mixer. Add 1).

3) Add the egg yolk.

4) Add the oil continuously in a vacuum.

5) After 2/3 of the oil has been added (slowly), blend the tarragon vinegar with the remaining 1/3 of the oil, and add.

The following data show that when AF is added to the mayonnaise as an anti-oxidant the results are comparable to the known food anti-oxididants GRINDOX 142 and GRINDOX 1029.

GRINDOX 142:

Ascorbyl palmitate	10%
Propyl gallate	20%
Citric acid	10%
Food grade emulsifier	60%
Form at 25°C	paste
Colour	grey to pale brown
Density	1.1 g/ml

(All percentages are by weight)

33

GRINDOX 1029:

Ascorbyl palmitate	20%
Natural tocopherols	20%
Food grade emulsifier	60%
Form at 25°C	paste
Colour	light brown
Density at 25°C	1,0 g/ml

(All percentages are by weight).

In the test procedure the anti-oxidants were added to the mayonnaise to provide an anti-oxidant concentration in the order of about 500 ppm. The mayonnaise was then placed in a bomb calorimeter at temperature 80°C containing pure O_2. An inductiom period to the onset of substantial oxidation of the product is then measured.

The results were as follows.

Samples:		IP (hours)
1.	Blank	28,0
2.	+ 500 ppm GRINDOX 142	35,0
3.	+ 500 ppm GRINDOX 1029	33,3
4.	+ 550 ppm GRINDOX 1029	34,3
5.	+ 500ppm 1,5 anhydro-D-fructose	32,0

(IP hours = Induction Period)

These results show that AF is an excellent food anti-oxidant and is comparable with the known foodstuffs anti-oxidants GRINDOX 142 or GRINDOX 1029.

34

EXAMPLE 4.2

Use of AF as an anti-oxidant in a salad dressing

5   **YOGURT SALAD DRESSING WITH 50% OIL**

Yogurt salad dressing with 50% oil is used for salads, potatoes, raw vegetable salad,
meat, fish and boiled vegetables.

10   **Composition**

Soya oil	50.0%
Yogurt (plain)	39.0%
Vinegar (10%)	3.5%
Sugar	3.0%
Egg yolk	2.0%
Salt	1.0%
Potassium sorbate	0.1%
MAYODAN 525	1.4%
Acid masking flavouring 2072	0.02%

20

MAYODAN 525 provides unique emulsion stability, prevents syneresis, ensures
uniform oil dispersion and viscosity, improves tolerance to production processes and
ensures a long shelf life.

25   Flavouring 2072 is a r·· :ure-identical, acid masking flavouring reducing the acidulated
taste of dressing without affecting its pH value.

Process

30   1.   Dry mix MAYODAN 525, sugar and salt. Disperse in oil in a ratio of 1 part
powder to 2 parts oil.

35

2.   Fill flavouring, potassium sorbate and yogurt into the Koruma mixer. Add 1).

3.   Add the egg yolk.

5   4.   Add the oil continuously in a vacuum.

5.   After 2/3 of the oil has been added (slowly), blend the vinegar with the remaining 1/3 of the oil, and add.

10   6.   Add spices if required.

Test results:

Sample:	IP hours	PF
1. Blank	37.2	1.00
15 2. 500 ppm anhydrofructose	39.5	1.06
3. 800 ppm GRINDOX 1032	43.3	1.07

(IP - Induction Period); (PF - Protection Period)

Protection Factor (PF):

20   For each temperature defined as

PF = IP of the oil with added antioxidant/IP of the same oil without added antioxidant

Life extension (LE) %:

25   LE = (PF - 1.0) x 100

36

6. PREPARATIONS OF α-1,4-GLUCAN LYASE

INTRODUCTION

5   With regard to a further embodiments of the present invention the enzyme α-1,4-
       glucan lyase for use in preparing the AF may be isolated from a fungally infected
       algae, preferably fungally infected *Gracilariopsis lemaneiformis*, more preferably
       fungally infected *Gracilariopsis lemaneiformis* from Qingdao (China).

10   Alternatively the enzyme may be obtained from a fungus. For example, the fungus
       can be any one of *Discina perlata, Discina parma, Gyromitra gigas, Gyromitra
       infula, Mitrophora hybrida, Morchella conica, Morchella costata, Morchella elata,
       Morchella hortensis, Morchella rotunda, Morchella vulgaris, Peziza badia,
       Sarcosphaera eximia, Disciotis venosa, Gyromitra esculenta, Helvella crispa, Helvella
15   lacunosa, Leptopodia elastica, Verpa digitaliformis*, and other forms of *Morchella*.
       Preferably the fungus is *Morchella costata* or *Morchella vulgaris*.

       With regard to a further embodiment of the present invention the enzyme α-1,4-
       glucan lyase for use in preparing the AF may be isolated from algae alone, preferably
20   *Gracilariopsis lemaneiformis*, more preferably *Gracilariopsis lemaneiformis* from
       Santa Cruz (California).

       The initial enzyme purification can be performed by the method as described by Yu
       et al (ibid). However, preferably, the initial enzyme purification includes an
25   optimized procedure in which a solid support is used that does not decompose under
       the purification step. This gel support further has the advantage that it is compatible
       with standard laboratory protein purification equipment. The details of this optimized
       purification strategy are given later on. The purification is terminated by known
       standard techniques for protein purification.

30

       The purity of the enzyme can be readily established using complementary
       electrophoretic techniques.

37

A.   SOURCE = FUNGALLY INFECTED ALGAE

The following sequence information was used to generate primers for the PCR
reactions mentioned below and to check the amino acid sequence generated by the
respective nucleotide sequences.

Amino acid sequence assembled from peptides from fungus infected *Gracilariopsis*
lemaneiformis

Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala

Ala Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asn Asn

Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly

Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu

Asn Ser Thr Glu Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp

Tyr Lys Phe Gly Pro Asp Phe Asp Thr Lys Pro Leu Glu Gly Ala

The Amino acid sequence (27-34) used to generate primer A and B (Met Tyr Asn
Asn Asp Ser Asn Val)

Primer A

ATG TA(TC) AA(CT) AA(CT) GA(CT) TC(GATC) AA(CT) GT   128 mix

Primer B

ATG TA(TC) AA(CT) AA(CT) GA(CT) AG(CT) AA(CT) GT   64 mix

The Amino acid sequence (45-50) used to generate primer C (Gly Gly His Asp Gly
Tyr)

Primer C

TA (GATC)CC (GA)TC (GA)TG (GATC)CC (GATC)CC   256 mix

[The sequence corresponds to the complementary strand.]

38

The Amino acid sequence (74-79) used to generate primer E (Gln Trp Tyr Lys Phe Gly)

Primer E

5   GG(GATC) CC(GA) AA(CT) TT(GA) TAC CA(CT) TG   64 mix

[The sequence corresponds to the complementary strand.]

The Amino acid sequence (1-6) used to generate primer F1 and F2 (Tyr Arg Trp Gln Glu Val)

10

Primer F1

TA(TC) CG(GATC) TGG CA(GA) GA(GA) GT   32 mix

Primer F2

15   TA(TC) AG(GA) TGG CA(GA) GA(GA) GT   16 mix

The sequence obtained from the first PCR amplification (clone 1)

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT

20   TCTTGGCGGC CACGACGGTT A

Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly

Gly His Asp Gly

25   The sequence obtained from the second PCR amplification (clone 1)

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT

TCTTGGTGGA CATGATGGAT ATCGCATTCT GTGCGCGCCT GTTGTGTGGG

AGAATTCGAC CGAACGNGAA TTGTACTTGC CCGTGCTGAC CCAATGGTAC

AAATTCGGCC C

30   Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly

Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu

Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro

39

The sequence obtained from the third PCR amplification (clone2)

TACAGGTGGC AGGAGGTGTT GTACACTGCT ATGTACCAGA

ATGCGGCTTT CGGGAAACCG ATTATCAAGG CAGCTTCCAT

GTACGACAAC GACAGAAACG TTCGCGGCGC ACAGGATGAC

5   CACTTCCTTC TCGGCGGACA CGATGGATAT CGTATTTTGT

GTGCACCTGT TGTGTGGGAG AATACAACCA GTCGCGATCT

GTACTTGCCT GTGCTGACCA GTGGTACAAA TTCGGCCC

Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala Phe Gly Lys

10   Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn Asp Arg Asn Val Arg Gly Ala Gln Asp

Asp His Phe Leu Leu Gly Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val

Trp Glu Asn Thr Thr Ser Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys

Phe Gly

15   ## A.1.   CYTOLOGICAL   INVESTIGATIONS   OF   *GRACILARIOPSIS* *LEMANEIFORMIS*

A.1.1.1   Detection of fungal infection in *Gracilariopsis lemaneiformis*

20   Sections of *Gracilariopsis lemaneiformis* collected in China were either hand cut or cut from paraffin embedded material. Sectioned material was carefully investigated by light microscopy. Fungal hyphae were clearly detected in *Gracilariopsis lemaneiformis*.

25   The thalli of the *Gracilariopsis lemaneiformis* are composed of cells appearing in a highly ordered and almost symmetric manner. The tubular thallus of G. lemaneiformis is composed of large, colourless central cells surrounded by elongated, slender, ellyptical cells and small, round, red pigmented peripherial cells. All algal cell types are characterized by thick cell walls. Most of the fungal hyphae are found

30   at the interphase between the central layer of large cells and the peripherial layer. These cells can clearly be distinguished from the algae cells as they are long and cylindrical. The growth of the hyphae is observed as irregularities between the highly

40

ordered algae cells. The most frequent orientation of the hypha is along the main axis of the algal thallus. Side branches toward the central and periphery are detected in some cases. The hypha can not be confused with the endo/epiphytic 2nd generation of the algae.

Calcofluor White is known to stain chitin and cellulose containing tissue. The reaction with chitin requires four covalently linked terminal n-acetyl glucosamine residues. It is generally accepted that cellulose is almost restricted to higher plants although it might occur in trace amounts in some algae. It is further known that chitin is absent in *Gracilaria*.

Calcofluor White was found to stain domains corresponding to fungi hyfa cell walls in sectioned *Gracilariopsis lemaneiformis* material.

The hypha appear clear white against a faint blue background of *Gracilaria* tissue when observed under u.v. light - see Figure 1. Chitin is the major cell wall component in most fungi but absent in *Gracilaria*. Based upon these observations we conclude that the investigated algae is infected by a fungi. 40% of the lower parts of the investigated *Gracilariopsis lemaneiformis* sections were found to be infected with fungal hyphae. In the algae tips 25% of the investigated *Gracilariopsis lemaneiformis* sections were found to be infected.

Staining of sectioned *Gracilariopsis lemaneiformis* with Periodic acid Schiff (PAS) and Aniline blue black revealed a significantly higher content of carbohydrates within the fungal cells as compared with the algae cells - see Figure 2. Safranin O and Malachit Green showed the same colour reaction of fungi cells as found in higher plants infected with fungi.

An Acridin Orange reaction with sectioned *Gracilariopsis lemaneiformis* showed clearly the irregularly growth of the fungus.

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A.1.1.2   Electron Microscopy

Slides with 15 μm thick sections, where the fungus was detected with Calcofluor White were fixed in 2% OsO_4, washed in water and dehydrated in dimethoxypropane and absolute alcohol. A drop of a 1:1 mixture of acetone and Spurr resin was placed over each section on the glass slide, and after one hour replaced by a drop of pure resin. A gelatin embedding capsule filled with resin was placed face down over the section and left over night at 4°C. After the polymerization at 55°C for 8 hrs, the thick sections adhering to the resin blocks could can be separated from the slide by immersion in liquid nitrogen.

Blocks were trimmed and 100 nm thick sections were cut using a diamond knife on a microtome. The sections were stained in aqueous uranyl acetate and in lead citrate. The sections were examined in an electron microscope at 80 kV.

The investigation confirmed the ligth microscopical observations and provided further evidence that the lyase producing, chinese strain of G. lamneiformis is infected by a fungal parasite or symbiont.

Fungal hyphae are build of tubular cells 50 to 100 μm long and only few microns in diameter. The cells are serially arranged with septate walls between the adjacent cells. Ocasional branches are also seen. The hyphae grow between the thick cell walls of algal thallus without penetrating the wall or damaging the cell. Such a symbiotic association, called mycophycobiosis, is known to occur between some filamentous marine fungi and large marine algae (Donk and Bruning, 1992 - Ecology of aquatic fungi in and on algae. In Reisser, W.(ed.): Algae and Symbioses: Plants, Animals, Fungi, Viruses, Interactions Explored. Biopress Ltd.,Bristol.)

Examining the microphotograph in Figure 10, several differences between algal and fungal cells can be noticed. In contrast to several μm thick walls of the alga, the fungal walls are only 100-200 nm thick. Plant typical organells as chloroplasts with thyllacoid membranes as well as floridean starch grains can be seen in algal cells, but

42

not in the fungus.

Intercellular connections of red algae are characterized by specific structures termed pit plugs, or pit connections The structures are prominent, electron dense cores and they are important features in algal taxonomy (Pueschel, C.M.: An expanded survey of the ultrastructure of Red algal pit plugs. J. Phycol. 25, 625, (1989)). In our material, such connections were frequently observed in the algal thallus, but never between the cells of the fungus.

A.1.2 *In situ* Hybridization experiments

In situ hybridization technique is based upon the principle of hybridization of an antisense ribonucotide sequence to the mRNA. The technique is used to visualize areas in microscopic sections where said mRNA is present. In this particular case the technique is used to localize the enzyme α-1,4-glucan lyase in sections of *Gracilariopsis lemaneiformis*.

A.1.2.1 Preparation of ^{35}S labelled probes for *In situ* hybridization

A 238 bp PCR fragment from a third PCR amplification - called clone 2 (see above) - was cloned into the pGEM-3Zf(+) Vector (Promega). The transcription of the antisense RNA was driven by the SP6 promotor, and the sense RNA by the T7 promotor. The Ribonuclease protection assay kit (Ambion) was used with the following modifications. The transcripts were run on a 6% sequencing gel to remove the unincorporated nucleotide and eluted with the elution buffer supplied with the T7RNA polymerase in vitro Transcription Kit (Ambion). The antisense transcript contained 23 non-coding nucleotides while the sense contained 39. For hybridization 10^7 cpm/ml of the ^{35}S labelled probe was used.

In situ hybridisation was performed essentially as described by Langedale et.al.(1988). The hybridization temperature was found to be optimal at 45°C. After washing at 45°C the sections were covered with KodaK K-5 photographic emulsion

43

and left for 3 days at 5°C in dark (Ref: Langedale, J.A., Rothermel, B.A. and Nelson, T. (1988). Genes and development 2: 106-115. Cold Spring Harbour Laboratory).

5   The *in situ* hybridization experiments with riboprobes against the mRNA of α-1,4-glucan lyase, show strong hybridizations over and around the hypha of the fungus detected in *Gracilariopsis lemaneiformis* - see Figures 4 and 5. This is considered a strong indication that the α-1,4-glucan lyase is produced. A weak random background reactions were detected in the algae tissue of both *Gracilariopsis*

10   *lemaneiformis*. This reaction was observed both with the sense and the antisense probes. Intense staining over the fungi hypha was only obtained with antisense probes.

These results were obtained with standard hybridisation conditions at 45°C in

15   hybridization and washing steps. At 50°C no staining over the fungi was observed, whereas the background staining remained the same. Raising the temperature to 55°C reduced the background staining with both sense and antisense probes significantly and equally.

20   Based upon the cytological investigations using complementary staining procedures it is concluded that *Gracilariopsis lemaneiformis* is fungus infected. The infections are most pronounced in the lower parts of the algal tissue.

In sectioned *Gracilariopsis lemaneiformis* material *in situ* hybridization results clearly

25   indicate that hybridization is restricted to areas where fungal infections are found - see Figure 4. The results indicate that α-1,4-glucan lyase mRNA appears to be restricted to fungus infected areas in *Gracilariopsis lemaneiformis*. Based upon these observations we conclude that α-1,4-glucan lyase activity is detected in fungally infected *Gracilariopsis lemaneiformis*.

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A.2. ENZYME PURIFICATION AND CHARACTERIZATION

Purification of α-1,4-glucan lyase from fungal infected *Gracilariopsis lemaneiformis* material was performed as follows.

A.2.1 Materials and Methods

The algae were harvested by filtration and washed with 0.9% NaCl. The cells were broken by homogenization followed by sonication on ice for 6x3 min in 50 mM citrate-NaOH pH 6.2 (Buffer A). Cell debris were removed by centrifugation at 25,000xg for 40 min. The supernatant obtained at this procedure was regarded as cell-free extract and was used for activity staining and Western blotting after separation on 8-25% gradient gels.

A.2.2 Separation by β-cyclodextrin Sepharose gel

The cell-free extract was applied directly to a β-cyclodextrin Sepharose gel 4B clolumn (2.6 x 18 cm) pre equilibrated with Buffer A. The column was washed with 3 volumes of Buffer A and 2 volumes of Buffer A containing 1 M NaCl. α-1,4-glucan lyase was eluted with 2 % dextrins in Buffer A. Active fractions were pooled and the buffer changed to 20 mM Bis-tris propane-HCl (pH 7.0, Buffer B).

Active fractions were applied onto a Mono Q HR 5/5 column pre-equilibrated with Buffer B. The fungal lyase was eluted with Buffer B in a linear gradient of 0.3 M NaCl.

The lyase preparation obtained after β-cyclodextrin Sepharose chromatography was alternatively concentrated to 150 μl and applied on a Superose 12 column operated under FPLC conditions.

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A.2.3 Assay for α-1,4-glucan lyase activity and conditions for determination of substrate specificity, pH and temperature optimum

The reaction mixture for the assay of the α-1,4-glucan lyase activity contained 10 mg

5   ml^{-1} amylopectin and 25 mM Mes-NaOH (pH 6.0). The reaction was carried out at 30°C for 30 min and stopped by the addition of 3,5-dinitrosalicylic acid reagent. Optical density at 550nm was measured after standing at room temperature for 10 min.

10   **A.3. AMINO ACID SEQUENCING OF THE α-1,4-GLUCAN LYASE FROM FUNGUS INFECTED *GRACILARIOPSIS LEMANEIFORMIS***

A.3.1 Amino acid sequencing of the lyases

15   The lyases were digested with either endoproteinase Arg-C from *Clostridium histolyticum* or endoproteinase Lys-C from *Lysobacter enzymogenes*, both sequencing grade purchased from Boehringer Mannheim, Germany. For digestion with endoproteinase Arg-C, freeze dried lyase (0.1 mg) was dissolved in 50 μl 10 M urea, 50 mM methylamine, 0.1 M Tris-HCl, pH 7.6. After overlay with N_2 and addition

20   of 10 μl of 50 mM DTT and 5 mM EDTA the protein was denatured and reduced for 10 min at 50°C under N_2. Subsequently, 1 μg of endoproteinase Arg-C in 10 μl of 50 mM Tris-HCl, pH 8.0 was added, N_2 was overlayed and the digestion was carried out for 6h at 37°C. For subsequent cysteine derivatization, 12.5 μl 100 mM iodoaceta-mide was added and the solution was incubated for 15 min at RT in the dark under

25   N_2.

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved in 50 μl of 8 M urea, 0.4 M NH_4HCO_3, pH 8.4. After overlay with N_2 and addition of 5 μl of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C

30   under N_2. After cooling to RT, 5 μl of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at RT in the dark under N_2.

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Subsequently, 90 μl of water and 5 μg of endoproteinase Lys-C in 50 μl of 50 mM tricine and 10 mM EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N$_2$.

5   The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 μm; The Separations Group; California) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 μm; Dr. Ole Schou, Novo Nordisk, Denmark) using the same solvent system prior to sequencing on an

10   Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

The amino acid sequence information from the enzyme derived from fungus infected *Gracilariopsis lemaneiformis* is shown below, in particular SEQ. ID. No. 1. and SEQ. ID. No. 2.

15

SEQ. I.D. No. 1 has:

Number of residues : 1088.

Amino acid composition (including the signal sequence)

61 Ala	15 Cys	19 His	34 Met	78 Thr
51 Arg	42 Gln	43 Ile	53 Phe	24 Trp
88 Asn	53 Glu	63 Leu	51 Pro	58 Tyr
79 Asp	100 Gly	37 Lys	62 Ser	77 Val

25   SEQ. I.D. No. 2 has:

Number of residues : 1091.

Amino acid composition (including the signal sequence)

58 Ala	16 Cys	14 His	34 Met	68 Thr
57 Arg	40 Gln	44 Ile	56 Phe	23 Trp
84 Asn	47 Glu	69 Leu	51 Pro	61 Tyr
81 Asp	102 Gly	50 Lys	60 Ser	76 Val

47

A.3.2 N-TERMINAL ANALYSIS

Studies showed that the N-terminal sequence of native glucan lyase 1 was blocked. Deblocking was achieved by treating glucan lyase 1 blotted onto a PVDF membrane with anhydrous TFA for 30 min at 40°C essentially as described by LeGendre et al. (1993) [Purification of proteins and peptides by SDS-PAGE; In: Matsudaira, P. (ed.) A practical guide to protein and peptide purification for microsequencing, 2nd edition; Academic Press Inc., San Diego; pp. 74-101.]. The sequence obtained was TALSDKQTA, which matches the sequence (sequence position from 51 to 59 of SEQ. I.D. No.1) derived from the clone for glucan lyase 1 and indicates N-acetylthreonine as N-terminal residue of glucan lyase 1. Sequence position 1 to 50 of SEQ. I.D. No. 1 represents a signal sequence.

A.4. DNA SEQUENCING OF GENES CODING FOR THE α-1,4-GLUCAN LYASE FROM FUNGUS INFECTED _GRACILARIOPSIS LEMANEIFORMIS_

A.4.1 METHODS FOR MOLECULAR BIOLOGY

DNA was isolated as described by Saunders (1993) with the following modification: The polysaccharides were removed from the DNA by ELUTIP-d (Schleicher & Schuell) purification instead of gel purification. (Ref:Saunders, G.W. (1993). Gel purification of red algal genomic DNA: An inexpensive and rapid method for the isolation of PCR-friendly DNA. Journal of phycology 29(2): 251-254 and Schleicher & Schuell: ELUTIP-d. Rapid Method for Purification and Concentration of DNA.)

A.4.2 PCR

The preparation of the relevant DNA molecule was done by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufactures instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

48

PCR cycles:

no of cycles	C	time (min.)
1	98	5
	60	5

addition of Taq polymerase and oil

35	94	1
	47	2
	72	3
1	72	20

A.4.3 CLONING OF PCR FRAGMENTS

PCR fragments were cloned into pT7Blue (from Novagen) following the instructions of the supplier.

A.4.4 DNA SEQUENCING

Double stranded DNA was sequenced essentially according to the dideoxy method of Sanger et al. (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson, A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.)

The sequences are shown as SEQ.I.D. No.s 1 and 2. In brief:

SEQ. I.D. No. 3 has:

Total number of bases: 3267.

DNA sequence composition: 850 A; 761 C; 871 G; 785 T

SEQ. I.D. No. 4 has:

Total number of bases: 3276.

DNA sequence composition: 889 A; 702 C; 856 G; 829 T

49

A.4.5 SCREENING OF THE LIBRARY

Screening of the Lambda Zap library obtained from Stratagene, was performed in accordance with the manufacturer's instructions except that the prehybridization and hybridization was performed in 2xSSC, 0.1% SDS, 10xDenhardt's and 100μg/ml denatured salmon sperm DNA. To the hybridization solution a 32P-labeled denatured probe was added. Hybridization was performed over night at 55°C. The filters were washed twice in 2xSSC, 0.1% SDS and twice in 1xSSC, 0.1% SDS.

A.4.6 PROBE

The cloned PCR fragments were isolated from the pT7 blue vector by digestion with appropriate restriction enzymes. The fragments were seperated from the vector by agarose gel electrophoresis and the fragments were purified from the agarose by Agarase (Boehringer Mannheim). As the fragments were only 90-240 bp long the isolated fragments were exposed to a ligation reaction before labelling with 32P-dCTP using either Prime-It random primer kit (Stratagene) or Ready to Go DNA labelling kit (Pharmacia).

A.4.7 RESULTS

A.4.7.1 Generation of PCR DNA fragments coding for α-1,4-glucan lyase.

The amino acid sequences of three overlapping tryptic peptides from α-1,4-glucan lyase were used to generate mixed oligonucleotides, which could be used as PCR primers (see the sequences given above).

In the first PCR amplification primers A/B (see above) were used as upstream primers and primer C (see above) was used as downstream primer. The size of the expected PCR product was 71 base pairs.

50

In the second PCR amplification primers A/B were used as upstream primers and E was used as downstream primer. The size of the expected PCR product was 161 base pairs.

5   In the third PCR amplification primers F1 (see above) and F2 (see above) were used as upstream primers and E was used as downstream primer. The size of the expected PCR product was 238 base pairs.

The PCR products were analysed on a 2% LMT agarose gel and fragments of the
10   expected sizes were cut out from the gel and treated with Agarase (Boehringer Manheim) and cloned into the pT7blue Vector (Novagen) and sequenced.

The cloned fragments from the first and second PCR amplification coded for amino acids corresponding to the sequenced peptides (see above). The clone from the third
15   amplification (see above) was only about 87% homologous to the sequenced peptides.

A.4.7.2 Screening of the genomic library with the cloned PCR fragments.

Screening of the library with the above-mentioned clones gave two clones. One clone
20   contained the nucleotide sequence of SEQ I.D. No. 4 (gene 2). The other clone contained some of the sequence of SEQ I.D. No.3 (from base pair 1065 downwards) (gene 1).

The 5' end of SEQ. I.D. No. 3 (i.e. from base pair 1064 upwards) was obtained by
25   the RACE (rapid amplification of cDNA ends) procedure (Michael, A.F., Michael, K.D. & Martin, G.R.(1988). Proc..Natl.Acad.Sci.USA 85:8998-99002.) using the 5' race system from Gibco BRL. Total RNA was isolated according to Collinge et al.(Collinge, D.B., Milligan D.E:, Dow, J.M., Scofield, G.& Daniels, M.J.(1987). Plant Mol Biol 8: 405-414). The 5' race was done according to the protocol of the
30   manufacturer, using 1μg of total RNA. The PCR product from the second ammplification was cloned into pT7blue vector from Novagen according to the protocol of the manufacturer. Three independent PCR clones were sequenced to

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compensate for PCR errors.

An additional PCR was performed to supplement the clone just described with XbaI
and NdeI restriction sites immediately in front of the ATG start codon using the
5   following   oligonucleotide   as   an   upstream   primer:
GCTCTAGAGCATGTTTTCAACCCTTGCG
and a primer containing the complement sequence of bp 1573-1593 in sequence GL1
(i.e. SEQ. I.D. No. 3) was used as a downstream primer.

10   The complete sequence for gene 1 (i.e. SEQ. I.D. No. 3) was generated by cloning
the 3' end of the gene as a BamHI-HindIII fragment from the genomic clone into the
pBluescript II KS+ vector from Stratagene and additionally cloning the PCR
generated 5' end of the gene as a XbaI-BamHI fragment in front of the 3' end.

15   Gene 2 was cloned as a HindIII blunt ended fragment into the EcoRV site of
pBluescript II SK+ vector from Stratagene. A part of the 3' untranslated sequence
was removed by a SacI digestion, followed by religation. HindIII and HpaI
restriction sites were introduced immediately in front of the start ATG by digestion
with HindIII and NarI and religation in the presence of the following annealed
20   oligonucleotides

AGCTTGTTAACATGTATCCAACCCTCACCTTCGTGG
        ACAATTGTACATAGGTTGGGAGTGGAAGCACCGC

25   No introns were found in the clones sequenced.

The clone 1 type (SEQ.ID.No.3) can be aligned with all ten peptide sequences (see
Figure 8) showing 100% identity. Alignment of the two protein sequences encoded
by the genes isolated from the fungal infected algae *Gracilariopsis lemaneiformis*
30   shows about 78% identity, indicating that both genes are coding for a α-1.4-glucan
lyase.

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A.5. EXPRESSION OF THE GL GENE IN MICRO-ORGANISMS

(E.G. ANALYSES OF *PICHIA* LYASE TRANSFORMANTS AND *ASPERGILLUS* LYASE TRANSFORMANTS)

5   The DNA sequence encoding the GL was introduced into microorganisms to produce
        an enzyme with high specific activity and in large quantities.

        In this regard, gene 1 (i.e. SEQ. I.D. No. 3) was cloned as a NotI-HindIII blunt
        ended (using the DNA blunting kit from Amersham International) fragment into the
10   *Pichia* expression vector pHIL-D2 (containing the AOX1 promoter) digested with
        EcoRI and blunt ended (using the DNA blunting kit from Amersham International)
        for expression in *Pichia pastoris* (according to the protocol stated in the *Pichia*
        Expression Kit supplied by Invitrogen).

15   In another embodiment, the gene 1 (i.e. SEQ. I.D. No. 3) was cloned as a NotI-
        HindIII blunt ended fragment (using the DNA blunting kit from Amersham
        International) into the Aspergillus expression vector pBARMTE1 (containing the
        methyl tryptophan resistance promoter from *Neuropera crassa*) digested with SmaI
        for expression in *Aspergillus niger* (Pall et al (1993) Fungal Genet Newslett. vol 40
20   pages 59-62). The protoplasts were prepared according to Daboussi et al (Curr Genet
        (1989) vol 15 pp 453-456) using lysing enzymes Sigma L-2773 and the lyticase Sigma
        L-8012. The transformation of the protoplasts was followed according to the protocol
        stated by Buxton et al (Gene (1985) vol 37 pp 207-214) except that for plating the
        transformed protoplasts the protocol laid out in Punt et al (Methods in Enzymology
25   (1992) vol 216 pp 447 - 457) was followed but with the use of 0.6% osmotic
        stabilised top agarose.

        The results showed that lyase activity was observed in the transformed *Pichia pastoris*
        and *Aspergillus niger*.

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A.5.1 GENERAL METHODS

Preparation of cell-free extracts.

5   The cells were harvested by centrifugation at 9000 rpm for 5 min and washed with
0.9% NaCl and resuspended in the breaking buffer (50mM K-phosphate, pH 7.5
containing 1mM of EDTA, and 5% glycerol). Cells were broken using glass beads
and vortex treatment. The breaking buffer contained 1 mM PMSF (protease inhibi-
tor). The lyase extract (supernatant) was obtained after centrifugation at 9000 rpm for
10   5 min followed by centrifugation at 20,000 xg for 5min.

Assay of lyase activity by alkaline 3,5-dinitrosalicylic acid reagent (DNS)

One volume of lyase extract was mixed with an equal volume of 4% amylopectin
15   solution. The reaction mixture was then incubated at a controlled temperature and
samples vere removed at specified intervals and analyzed for AF.

The lyase activity was also analyzed using a radioactive method.

20   The reaction mixture contained 10 μl ^{14}C-starch solution (1 μCi; Sigma Chemicals
Co.) and 10 μl of the lyase extract. The reaction mixture was left at 25°C overnight
and was then analyzed in the usual TLC system. The radioactive AF produced was
detected using an Instant Imager (Pachard Instrument Co., Inc., Meriden, CT).

25   Electrophoresis and Western blotting

SDS-PAGE was performed using 8-25% gradient gels and the PhastSystem
(Pharmacia). Western blottings was also run on a Semidry transfer unit of the
PhastSystem.

30

Primary antibodies raised against the lyase purified from the red seaweed collected
at Qingdao (China) were used in a dilution of 1:100. Pig antirabbit IgG conjugated

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to alkaline phosphatase (Dako A/S, Glostrup, Denmark) were used as secondary antibodies and used in a dilution of 1:1000.

Part I, Analysis of the Pichia transformantscontaining the above mentioned construct

Results:

1. Lyase activity was determined 5 days after induction (according to the manual) and proved the activity to be intracellular for all samples in the B series.

Samples of B series:	11	12	13	15	26	27	28	29	30

Specific activity:	139	81	122	192	151	253	199	198	150

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*Specific activity is defined as nmol AF released per min per mg protein in a reaction mixture containing 2% (w/v) of glycogen, 1% (w/v) glycerol in 10 mM potassium phosphate buffer (pH 7.5). The reaction temperature was 45°C; the reaction time was 60 min.

A time course of sample B27 is as follows. The data are also presented in Figure 1.

Time (min)	0	10	20	30	40	50	60

Spec. act.	0	18	54	90	147	179	253

Assay conditions were as above except that the time was varied.

2. Western-blotting analysis.

The CFE of all samples showed bands with a molecular weight corresponding to the native lyase.

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MC-Lyase expressed intracellularly in *Pichia pastoris*

Names of culture   Specific activity*

A18   10

A20   32

A21   8

A22   8

A24   6

Part II, The *Aspergilus* transformants

Results

I. Lyase activity was determined after 5 days incubation(minimal medium containing 0.2% casein enzymatic hydrolysate analysis by the alkaline 3,5-dinitrosalicylic acid reagent

1). Lyase activity analysis of the culture medium

Among 35 cultures grown with 0.2% amylopectin included in the culture medium, AF was only detectable in two cultures. The culture medium of 5.4+ and 5.9+ contained 0.13 g AF/liter and 0.44 g/liter, respectively. The result indicated that active lyase had been secreted from the cells. Lyase activity was also measurable in the cell-free extract.

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2). Lyase activity analysis in cell-free extracts

Name of the culture	Specific activity*
5.4+	51
5.9+	148
5.13	99
5.15	25
5.19	37

*The specific activity was defined as nmol of AF produced per min per mg protein at 25°C. + indicates that 0.2% amylopectin was added.

The results show that Gene 1 of GL was expressed intracellular in *A. niger*.

Experiments with transformed E.coli (using cloning vectors pQE30 from the Qia express vector kit from Qiagen) showed expression of enzyme that was recognised by anti-body to the enzyme purified from fungally infected *Gracilariopsis lemaneiformis*.

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B.   **SOURCE = FUNGUS**

B.1. ENZYME PURIFICATION AND CHARACTERIZATION OF THE α-1,4-GLUCAN LYASE FROM THE FUNGUS _MORCHELLA COSTATA_

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B.1.1 Materials and Methods

The fungus _Morchella costata_ was obtained from American Type Culture Collection (ATCC). The fungus was grown at 25°C on a shaker using the culture medium recommended by ATCC. The mycelia were harvested by filtration and washed with 0.9% NaCl.

The fungal cells were broken by homogenization followed by sonication on ice for 6x3 min in 50 mM citrate-NaOH pH 6.2 (Buffer A). Cell debris were removed by centrifugation at 25,000xg for 40 min. The supernatant obtained at this procedure was regarded as cell-free extract and was used for activity staining and Western blotting after separation on 8-25% gradient gels.

B.1.2 Separation by β-cyclodextrin Sepharose gel

The cell-free extract was applied directly to a β-cyclodextrin Sepharose gel 4B clolumn (2.6 x 18 cm) pre equilibrated with Buffer A. The column was washed with 3 volumes of Buffer A and 2 volumes of Buffer A containing 1 M NaCl. α-1,4-glucan lyase was eluted with 2 % dextrins in Buffer A. Active fractions were pooled and the buffer changed to 20 mM Bis-tris propane-HCl (pH 7.0, Buffer B).

Active fractions were applied onto a Mono Q HR 5/5 column pre-equilibrated with Buffer B. The fungal lyase was eluted with Buffer B in a linear gradient of 0.3 M NaCl. The lyase preparation obtained after β-cyclodextrin Sepharose chromatography was alternatively concentrated to 150 μl and applied on a Superose 12 column operated under FPLC conditions.

58

B.1.3 Assay for α-1,4-glucan lyase activity and conditions for determination of substrate specificity, pH and temperature optimum

The reaction mixture for the assay of the α-1,4-glucan lyase activity contained 10 mg
5   ml^{-1} amylopectin and 25 mM Mes-NaOH (pH 6.0).

The reaction was carried out at 30 °C for 30 min and stopped by the addition of 3,5-dinitrosalicylic acid reagent. Optical density at 550nm was measured after standing at room temperature for 10 min. 10 mM EDTA was added to the assay mixture
10   when cell-free extracts were used.

The substrate amylopectin in the assay mixture may be replaced with other substrates and the reaction temperature may vary as specified in the text.

15   In the pH optimum investigations, the reaction mixture contained amylopection or maltotetraose 10 mg ml^{-1} in a 40 mM buffer. The buffers used were glycine-NaOH (pH 2.0-3.5), HoAc-NaoAc (pH 3.5-5.5), Mes-NaOH (pH 5.5-6.7), Mops-NaOH (6.0-8.0) and bicine-NaOH (7.6-9.0). The reactions were carried out at 30°C for 30 min. The reaction conditions in the temperature optimum investigations was the same
20   as above except that the buffer Mops-NaOH (pH 6.0) was used in all experiments. The reaction temperature was varied as indicated in the text.

SDS-PAGE, Native-PAGE and isoelectrofocusing were performed on PhastSystem (Pharmacia, Sweden) using 8-25% gradient gels and gels with a pH gradient of 3-9,
25   respectively. Following electrophoresis, the gels were stained by silver staining according to the procedures recommended by the manufacturer (Pharmacia). The glycoproteins were stained by PAS adapted to the PhastSystem. For activity staining, the electrophoresis was performed under native conditions at 6°C.

30   Following the electrophoresis, the gel was incubated in the presence of 1% soluble starch at 30°C overnight. Activity band of the fungal lyase was revealed by staining with I$_2$/KI solution.

59

B.1.4 Results

B.1.4.1 Purification, molecular mass and isoelectric point of the α-1,4-glucan lyase

5   The fungal lyase was found to adsorb on columns packed with β-cyclodextrin
Sepharose, starches and Red Sepharose.   Columns packed with β-cyclodextrin
Sepharose 4B gel and starches were used for purification purposes.

The lyase preparation obtained by this step contained only minor contaminating
10   proteins having a molecular mass higher than the fungal lyase. The impurity was
either removed by ion exchange chromatography on Mono Q HR 5/5 or more effici-
ently by gel filtration on Superose 12.

The purified enzyme appeared colourless and showed no absorbance in the visible
15   light region. The molecular mass was determined to 110 kDa as estimated on SDS-
PAGE.

The purified fungal lyase showed a isoelectric point of pI 5.4 determined by
isoelectric focusing on gels with a pH gradient of 3 to 9.   In the native
20   electrophoresis gels, the enzyme appeared as one single band. This band showed
starch-degrading activity as detected by activity staining. Depending the age of the
culture from which the enzyme is extracted, the enzyme on the native and isoelectric
focusing gels showed either as a sharp band or a more diffused band with the same
migration rate and pI.

25

B.1.4.2 The pH and temperature optimum of the fungal lyase catalayzed reaction

The pH optimum pH range for the fungal lyase catalyzed reaction was found to be
between pH 5 and pH 7.

30

60

B.1.4.3 Substrate specificity

The purified fungal lyase degraded maltosaccharides from maltose to maltoheptaose.
However, the degradation rates varied. The highest activity achieved was with
maltotetraose (activity as 100%), followed by maltohexaose (97%), maltoheptaose
(76%), maltotriose (56%) and the lowest activity was observed with maltose (2%).

Amylopectin, amylose and glycogen were also degraded by the fungal lyase (% will
be determined). The fungal lyase was an exo-lyase, not a endolyase as it degraded
p-nitrophenyl α-D-maltoheptaose but failed to degrade reducing end blocked p-
nitrophenyl α-D-maltoheptaose.

B.1.5 *Morchella Vulgaris*

The protocols for the enzyme purification and charaterisation of alpha 1,4-glucal lyase
obtained from *Morchella Vulgaris* were the same as those above for *Morchella
Costata* (with similar results).

B.2. AMINO ACID SEQUENCING OF THE α-1,4-GLUCAN LYASE FROM
FUNGUS

B.2.1 Amino acid sequencing of the lyases

The lyases were digested with either endoproteinase Arg-C from *Clostridium
histolyticum* or endoproteinase Lys-C from *Lysobacter enzymogenes*, both sequencing
grade purchased from Boehringer Mannheim, Germany. For digestion with
endoproteinase Arg-C, freezedried lyase (0.1 mg) was dissolved in 50 μl 10 M urea,
50 mM methylamine, 0.1 M Tris-HCl, pH 7.6. After overlay with N_2 and addition
of 10 μl of 50 mM DTT and 5 mM EDTA the protein was denatured and reduced for
10 min at 50°C under N_2. Subsequently, 1 μg of endoproteinase Arg-C in 10 μl of 50
mM Tris-HCl, pH 8.0 was added, N_2 was overlayed and the digestion was carried out
for 6h at 37°C.

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For subsequent cysteine derivatization, 12.5 μl 100 mM iodoacetamide was added and the solution was incubated for 15 min at RT in the dark under N_2.

For digestion with endoproteinase Lys-C, freeze dried lyase (0.1 mg) was dissolved
5   in 50 μl of 8 M urea, 0.4 M NH_4HCO_3, pH 8.4. After overlay with N_2 and addition of 5 μl of 45 mM DTT, the protein was denatured and reduced for 15 min at 50°C under N_2. After cooling to RT, 5 μl of 100 mM iodoacetamide was added for the cysteines to be derivatized for 15 min at RT in the dark under N_2. Subsequently, 90 μl of water and 5 μg of endoproteinase Lys-C in 50 μl of 50 mM tricine and 10 mM
10   EDTA, pH 8.0, was added and the digestion was carried out for 24h at 37°C under N_2.

The resulting peptides were separated by reversed phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 μm; The Separations Group; California) using solvent A:
15   0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm; 3 μm; Dr. Ole Schou, Novo Nordisk, Denmark) using the same solvent system prior to sequencing on an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles.

20   The amino acid sequence information from the enzyme derived from the fungus *Morchella costata* is shown Fig. 17.

The amino acid sequence information from the enzyme derived from the fungus *Morchella vulgaris* is shown Fig. 18.
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B.3. DNA SEQUENCING OF GENES CODING FOR THE α-1,4-GLUCAN LYASE FROM FUNGUS

B.3.1 METHODS FOR MOLECULAR BIOLOGY
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DNA was isolated as described by Dellaporte et al (1983 - Plant Mol Biol Rep vol 1 pp19-21).

62

B.3.2 PCR

The preparation of the relevant DNA molecule was done by use of the Gene Amp DNA Amplification Kit (Perkin Elmer Cetus, USA) and in accordance with the manufactures instructions except that the Taq polymerase was added later (see PCR cycles) and the temperature cycling was changed to the following:

PCR cycles:

no of cycles	C	time (min.)
1	98	5
	60	5
addition of Taq polymerase and oil		
35	94	1
	47	2
	72	3
1	72	20

B.3.3 CLONING OF PCR FRAGMENTS

PCR fragments were cloned into pT7Blue (from Novagen) following the instructions of the supplier.

B.3.4 DNA SEQUENCING

Double stranded DNA was sequenced essentially according to the dideoxy method of Sanger et al. (1979) using the Auto Read Sequencing Kit (Pharmacia) and the Pharmacia LKB A.L.F.DNA sequencer. (Ref: Sanger, F., Nicklen, S. and Coulson, A.R.(1979). DNA sequencing with chain-determinating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463-5467.)

63

B.3.5 SCREENING OF THE LIBRARIES

Screening of the Lambda Zap libraries obtained from Stratagene, was performed in accordance with the manufacturer's instructions except that the prehybridization and hybridization was performed in 2xSSC, 0.1% SDS, 10xDenhardt's and 100μg/ml denatured salmon sperm DNA.

To the hybridization solution a 32P-labeled denatured probe was added. Hybridization was performed over night at 55°C. The filters were washed twice in 2xSSC, 0.1% SDS and twice in 1xSSC, 0.1% SDS.

B.3.6 PROBE

The cloned PCR fragments were isolated from the pT7 blue vector by digestion with appropriate restriction enzymes. The fragments were seperated from the vector by agarose gel electrophoresis and the fragments were purified from the agarose by Agarase (Boehringer Mannheim). As the fragments were only 90-240 bp long the isolated fragments were exposed to a ligation reaction before labelling with 32P-dCTP using either Prime-It random primer kit (Stratagene) or Ready to Go DNA labelling kit (Pharmacia).

B.3.7 RESULTS

B.3.7.1 Generation of PCR DNA fragments coding for α-1,4-glucan lyase.

The amino acid sequences (shown below) of three overlapping tryptic peptides from α-1,4-glucan lyase were used to generate mixed oligonucleotides, which could be used as PCR primers for amplification of DNA isolated from both MC and MV.

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Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr Val Leu Asp Ile Val Lys

Pro Gly His Gly Glu Tyr Val Gly Trp Gly Glu Met Gly Gly Ile Gln Phe Met Lys

Glu Pro Thr Phe Met Asn Tyr Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr

Ala Gln Gly Ala Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr

5

In the first PCR amplification primers A1/A2 (see below) were used as upstream
primers and primers B1/B2 (see below) were used as downstream primer.

10
Primer A1: CA(GA)CA(CT)AA(GA)ATGCT(GATC)AA(GA)GA(CT)AC

Primer A2: CA(GA)CA(CT)AA(GA)ATGTT(GA)AA(GA)GA(CT)AC

Primer B1: TA(GA)AA(GATC)GG(GA)TC(GA)CT(GA)TG(GA)TA

Primer B2: TA(GA)AA(GATC)GG(GA)TC(GATC)GA(GA)TG(GA)TA

The PCR products were analysed on a 2% LMT agarose gel and fragments of the
15   expected sizes were cut out from the gel and treated with Agarase (Boehringer
Manheim) and cloned into the pT7blue Vector (Novagen) and sequenced.

The cloned fragments from the PCR amplification coded for amino acids
corresponding to the sequenced peptides (see above) and in each case in addition to
20   two intron sequences. For MC the PCR amplified DNA sequence corresponds to the
sequence shown as from position 1202 to position 1522 with reference to Figure 14.
For MV the PCR amplified DNA sequence corresponds to the sequence shown as
from position 1218 to position 1535 with reference to Figure 15.

25   B.3.7.2 Screening of the genomic libraries with the cloned PCR fragments.

Screening of the libraries with the above-mentioned clone gave two clones for each
source. For MC the two clones were combined to form the sequence shown in
Figure 14 (see below). For MV the two clones could be combined to form the
30   sequence shown in Figure 15 in the manner described above.

65

An additional PCR was performed to supplement the MC clone with PstI, PvuII, AscI and NcoI restriction sites immediately in front of the ATG start codon using the following oligonucleotide as an upstream primer:

AAACTGCAGCTGGCGCGCCATGGCAGGATTTTCTGAT

5   and a primer containing the complement sequence of bp 1297-1318 in Figure 4 was used as a downstream primer.

The complete sequence for MC was generated by cloning the 5' end of the gene as a BglII-EcoRI fragment from one of the genomic clone (first clone) into the BamHI-

10   EcoRI sites of pBluescript II KS+ vector from Stratagene. The 3' end of the gene was then cloned into the modified pBluescript II KS+ vector by ligating an NspV (blunt ended, using the DNA blunting kit from Amersham International)-EcoRI fragment from the other genomic clone (second clone) after the modified pBluescript II KS+ vector had been digested with EcoRI and EcoRV. Then the intermediate part

15   of the gene was cloned in to the further modified pBluescript II KS+ vector as an EcoRI fragment from the first clone by ligating that fragment into the further modified pBluescript II KS+ vector digested with EcoRI.

B.4. EXPRESSION OF THE GL GENE IN MICRO-ORGANISMS

20

The DNA sequence encoding the GL can be introduced into microorganisms to produce the enzyme with high specific activity and in large quantities.

In this regard, the MC gene (Figure 14) was cloned as a XbaI-XhoI blunt ended

25   (using the DNA blunting kit from Amersham International) fragment into the *Pichia* expression vector pHIL-D2 (containing the AOX1 promoter) digested with EcoRI and blunt ended (using the DNA blunting kit from Amersham International) for expression in *Pichia pastoris* (according to the protocol stated in the *Pichia* Expression Kit supplied by Invitrogen).

30

66

In another embodiment, the MC gene 1 (same as Figure 14 except that it was modified by PCR to introduce restriction sites as described above) was cloned as a PvuII-XhoI blunt ended fragment (using the DNA blunting kit from Amersham International) into the Aspergillus expression vector pBARMTE1 (containing the methyl tryptophan resistance promoter from *Neuropera crassa*) digested with SmaI for expression in *Aspergillus niger* (Pall et al (1993) Fungal Genet Newslett. vol 40 pages 59-62). The protoplasts were prepared according to Daboussi et al (Curr Genet (1989) vol 15 pp 453-456) using lysing enzymes Sigma L-2773 and the lyticase Sigma L-8012. The transformation of the protoplasts was followed according to the protocol stated by Buxton et al (Gene (1985) vol 37 pp 207-214) except that for plating the transformed protoplasts the protocol laid out in Punt et al (Methods in Enzymology (1992) vol 216 pp 447 - 457) was followed but with the use of 0.6% osmotic stabilised top agarose.

The results showed that lyase activity was observed in the transformed *Pichia pastoris* and *Aspergillus niger*.

ANALYSES OF PICHIA LYASE TRANSFORMANTS AND *ASPERGILLUS* LYASE TRANSFORMANTS

GENERAL METHODS

Preparation of cell-free extracts.

The cells were harvested by centrifugation at 9000 rpm for 5 min and washed with 0.9% NaCl and resuspended in the breaking buffer (50mM K-phosphate, pH 7.5 containing 1mM of EDTA, and 5% glycerol). Cells were broken using glass beads and vortex treatment. The breaking buffer contained 1 mM PMSF (protease inhibitor). The lyase extract (supernatant) was obtained after centrifugation at 9000 rpm for 5 min followed by centrifugation at 20,000 xg for 5min.

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Assay of lyase activity by alkaline 3,5-dinitrosalicylic acid reagent (DNS)

One volume of lyase extract was mixed with an equal volume of 4% amylopectin solution. The reaction mixture was then incubated at a controlled temperature and samples were removed at specified intervals and analyzed for AF.

The lyase activity was also analyzed using a radioactive method.

The reaction mixture contained 10 μl ^{14}C-starch solution (1 μCi; Sigma Chemicals Co.) and 10 μl of the lyase extract. The reaction mixture was left at 25°C overnight and was then analyzed in the usual TLC system. The radioactive AF produced was detected using an Instant Imager (Pachard Instrument Co., Inc., Meriden, CT).

Electrophoresis and Western blotting

SDS-PAGE was performed using 8-25% gradient gels and the PhastSystem (Pharmacia). Western blottings was also run on a Semidry transfer unit of the PhastSystem. Primary antibodies raised against the lyase purified from the red seaweed collected at Qingdao (China) were used in a dilution of 1:100. Pig antirabbit IgG conjugated to alkaline phosphatase (Dako A/S, Glostrup, Denmark) were used as secondary antibodies and used in a dilution of 1:1000.

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Part I, Analysis of the Pichia transformantscontaining the above mentioned construct

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MC-Lyase expressed intracellularly in *Pichia pastoris*

Names of culture	Specific activity*
A18	10
A20	32
A21	8
A22	8
A24	6

*The specific activity was defined as nmol of AF produced per min per mg protein at 25°C.

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Part II, The *Aspergilus* transformants

Results

5   I. Lyase activity was determined after 5 days incubation(minimal medium containing
0.2% casein enzymatic hydrolysate analysis by the alkaline 3,5-dinitrosalicylic acid
reagent

Lyase activity analysis in cell-free extracts

10   --

Name of the culture	Specific activity*
8.13	11
8.16	538
8.19	37

----

*The specific activity was defined as nmol of AF produced per min per mg protein
20   at 25°C.

The results show that the MC-lyase was expressed intracellular in *A. niger*.

II. Lyase activity test by radioactive method
25

The cell-free extracts of the following cultures contained ^{14}C labelled AF

51+, 54+, 55+, 59+, 512, 513, 514, 515, 516, 518, 519.

The TLC of the degradation products of the α-1,4-glucan lyase reaction using ^{14}C-
30   starch as substrate are shown in Figure 20. The reaction mixture was applied on the
TLC. The lane number corresponds to the name of the culture: 1, 512; 2, 513; 3,
514; 4, 515; 5, 516; 6, 517; 7, 518; 8, 519; 9, 520. The fast moving spots are AF.

70

C. SOURCE = ALGAE ALONE

The protocols for the enzyme purification and charaterisation of alpha 1,4-glucal lyase obtained from *Gracilarioposis lemaneiformis* (as obtained from Santa Cruz) were essentially the same as those described above for, for example, *Morchella Costata* (with similar results).

1. Characterization of α-1,4-glucan lyase from the parasite-free red seaweed *Gracilariopsis lemaneiformis* collected in California.

The amino acid composition of the lyase is given in the following table.

Amino acid residues	mol % of each residue
Asx	15.42
Thr	5.24
Ser	6.85
Glx	9.46
Pro	5.46
Gly	9.08
Ala	5.38
1/2Cys	1.57
Val	6.60
Met	2.90
Ile	3.66
Leu	6.00
Tyr	6.00
Phe	4.37
His	1.65
Lys	4.44
Arg	4.17
Trp	1.75
Total:	100.00

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2. SEQUENCE ANALYSIS

Comparison of the peptide sequences from the Californian algae with the amino acid sequence from the fungally infected algae from China showed a high degree of homology (78 to 80% identity between the amino acid sequence generated from the PCR fragments and the corresponding sequences in the GL obtained from the algae from China) between the two protein sequences.

Three Oligonucleotides was generated from these two sequences from the Californian algae to generate a PCR fragment of app. 970 bp.

Primer 1: ATGAC(GATC)AA(CT)TA(CT)AA(CT)TA(CT)GA(CT)AA

Primer 2: (AG)TG(GATC)GGCATCAT(GATC)GC(GATC)GG(GATC)AC

Primer 3: GTCAT(GA)TC(CT)TGCCA(GATC)AC(GA)AA(GA)TC

Primer 1 was used as the upstream primer and primer 2 was used as the downstream primer in the first PCR amplification. In the second PCR amplification primer 1 was used as the upstream primer and primer 3 was used as the downstream primer. A PCR fragment of the expected size was generated and cloned into the pT7blue vector from Novagen. Three independent plasmids containing a PCR fragment were sequenced and it was seen that these three cloned PCR fragments contained the codons for peptide sequences originating from three different proteins. This indicates that there are at least three different genes coding for α-1,4-glucan lyase in the Californian algae.

3. The substrate concentration at which half of the maximal velocity rate was reached is 3.76 mg/ml for amylopectin and 3.37 mg/ml for glycogen.

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4. The degradation rates of the lyase on various substrates are given below.

Substrate	AF released (nmol)

5 Maltose   657

   Maltotriose   654

   Maltotetraose   670

10 Maltopentaose   674

   Maltohexaose   826

15 Maltoheptaose   865

   Dextrin 20   775

   Dextrin 15   775

20 Dextrin 10   844

   Amylopectin   732

25 Glycogen   592

Reaction conditions: The reaction mixture contained 10 mM of HOAc-NaOAc (pH 3.8). The substrate concentration was 10 mg/ml. The final volume was 100 ul after

30 the addition of lyase and water. The reaction time was 40 min at 45°C.

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The lyase was not able to degrade pullulan, nigeran tetrasaccharide, trehalose, isomaltose, glucose, α-, ß- and r-cyclodextrins. The lyase degraded panose and nigerose though at a slow rate.

5. 5. The temperature optimum for the lyase was 48°C when amylopectin was used as substrate and 50°C when glycogen was used as substrate. At 50°C, the reactivity of glycogen was similar to that of amylopectin; below 50°C, amylopectin was a better substrate than glycogen.

10. 6. The pH optimum range for the lyase was between pH 3.5 and pH 7.0; the optimal pH was 3.8. The buffers used in the pH tests were glycine-HCl (pH 2.2-3.6); NaOAc-HOAc (pH 3.5-5.5); Mes-NaOH (pH 5.5-6.7); Mops-NaOH (pH 6.0-8.0) and bicine-NaOH (pH 7.6-9.0). All buffers used were 40 mM.

15. 7. At a final concentration of 2 mM, p-chloromercuribenzoic acid (PCMB) inhibited the lyase activity by 96%, indicating the -SH group(s) is essential for the enzymatic activity.

7. FURTHER STUDIES

20.

7.1 Effect of alcohols in increasing the activity and stability of the lyase purified from the fungal infected algae.

1-propanol, 2-propanol and 1-butanol were tested at the following concentrations (0%,
25. 1%, 5% and 10%). The optimal concentration of 1-propanol was 5% which increased the AF yield by 34% after 6 days of incubation; the optimal concentration for 2-propanol was 1% which increased the AF yield by 20% after 10 days incubation; the optimal concentration for 1-butanol was 5% which increased the AF yield by 52% after 3-day incubation.

30.

Ethanol was tested at the following concentrations (0, 1, 3, 5, 7, 9, 11, 13, 15%). The optimal concentration for 7 days incubation was 5% which increased the AF

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yield by 12%. For 10 days incubation the optimal concentration was 3% which in-
creased AF yield by 16%.

The effect of 1-propanol:

1-propanol concentraction (v/v)	0	Reaction time (days)			
		1	3	6	10
		AF yield (μmol)			
0%	0	84	261	451	689
1%	0	80	280	530	803
5%	0	115	367	605	853
10%	0	107	307	456	583

7.2 Effect of different reaction media upon the production of AF by the lyase purified
from the fungal infected algae and the fugnal lyase from *M. costata* and *M. vulgaris*.

2.1. The lyase from the fungal infected algae.

The results (see table below) indicate that the best reaction medium is 5 mM of
HOAc-NaOAc (pH 3.9) (BACE for short) and containing mM concentrations of Na_2-
EDTA. The production of AF using either pure water or 0.85% NaCl as reaction
medium decreased the yield. Inclusion of 0.85% of NaCl in BACE also decreased the
AF yield.

Reaction Media	Reaction Time (days)			
	0	1	3	8
	AF yield (μmol)			
BACE	0	229	498	575
Water	0	46	128	217
NaCl (0.85%)	0	123	239	249
BACE+NaCl (0.85%)	0	153	281	303

75

2.2. The following buffers: Mes-NaOH, Mops-NaOH, Hepes-NaOH, and Bicine-NaOH were the optimal reaction media for the lyase from *M. costata* and -*M. vulgaris*. In the HOAc-NaOAc buffer, the lyase was unstable and therefore use of this buffer system caused a decrease in AF yield.

7.3. The effect of endoamylases and debranching enzymes upon the AF production.

3.1. The effect of endoamylase

The starch used for AF production may first be liquified either by endoamylases, or by acid hydrolysis.

Endoamylase degraded starch is more suitable as substrate for the lyase as compared to native starch. Starch has a limited solubility at the temperature used for the lyase-catalyzed reaction. Treatment of starch with endoamylases led to increased glucose yied. It was found that a reducing matter of around 10-15% (on a dry mater basis) was most suitable as substrate for the lyase with respect to AF yield and further treatment with the endoamylase to a reducing matter of 19% was no longer suitable for the lyase.

3.2. The effect of pullulanase and isoamylase

As seen from the results below, both the isoamylase and the pullulanase increased AF yield by up to 50% at pH 4.5 and 5.0. The reaction system consisted of the lyase from the fungal affected red algae with or without the addition of isoamylase or pullulanase (MegaZyme Ltd.). Amylopectin was used as substrate. The AF produced in the presence of only the lyase was expressed as 100%.

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The pH of the reaction medium			
Enzymes added	3.5	4.5	5.0
Lyase only	100	100	100
Lyase + isoamylase	136	152	150
Lyase + pullulanase	132	158	155

4. The relative degrading rates of the fungal lyase towards various substrates

4.1. The lyase from *M. costata*.

The activity observed with maltotetraose is expressed as 100%.

Substrate concentration	2mg/ml	4mg/ml	10mg/ml
Maltose	0.5	1.6	2.2
Maltotriose	40.6	58.6	56.0
Maltotetraose	100	100	100
Maltopentaose	107.1	100.1	99.7
Maltohexaose	86.6	98.2	95.9
Maltoheptaose	82.2	81.5	75.7
Dextrin 10*	-**	-	68.3
Dextrin 15*	-	-	61.1
Dextrin 20*	-	-	46.6
Soluble Starch	-	-	92.9
Amylopectin	-	-	106.5
glycogen	-	-	128.5

* the number indicates the contents of the reducing matter on a dry weight basis. **, not determined.

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4.2. The lyase from *M. vulgaris* lyase.

The activity observed for maltotetraose is treated as 100%. The final concentration of all substrates was 10 mg ml^{-1}.

Substrates	Activity (%)
Maltose	10.1
Maltotriose	49.8
Maltotetraose	100.0
Maltopentaose	79.3
Maltohexaose	92.4
Maltoheptaose	73.9
Dextrin 10	62
Dextrin 15	45
Dextrin 20	37
Soluble starch	100.5
Amylopectin	139.9
Glycogen	183.3

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The lyase from *M. costata* and *M. vulgaris* was unable to degrade the following sugars.

Trehalose, panose, nigerose, nigerotetraose, glucose, isomaltose, alpha-, beta and gama-cyclodextrins, pullulalans and non-reducing end blocked p-nitrophenyl α-D-maltoheptaoside as there was no AF detectable on a TLC plates after these substrates had been incubated for 48 h with the fungal lyase.

7.5. pH and temperature optimum for the lyase catalyzed reaction.

GL sources	Optimal pH	Optimal pH range	Optimal temperature
M. costata	6.5	5.5-7.5	37 C; 40 C*
M. vulgaris	6.4	5.9-7.6	43 C; 48 C*
Fungal infected *Gracilariopsis*			
lemaneiformis	3.8	3.7-4.1	40 C; 45 C*

*Parameters determined using glycogen as substrate; other parameters determined using amylopectin as substrate.

7.6. The stabilizing effect of glycogen on the lyase from the fungal infected Gracilariopsis lemaneiformis.

The results indicate that at higher temperatures the reaction rates were higher when glycogen was used as substrate instead of amylopectin.

	Reaction temperature		
Substrates	25 C	30 C	45 C
Amylopectin	0.818*	1.133*	1.171*
Glycogen	0.592*	0.904*	1.861*
The ratio of relative reaction rates between Glycogen and Amylopectin (%)			
	72.4	79.8	158.9

*, the relative reaction rates.

7.7. The molecular masses and pI values of the lyases

The molecular masses of the lyases from the fungal infected G. lemaneiformis, both forms of lyase from apparent fungal free G. lemaneiformis, from M. costata and M. vulgaris were estimated to 110,000 ± 10,000 daltons usind SDS-PAGE on a gradient gel (8-25%).

The pI of the lyase from the fungal infected G. lemaneiformis was around 3.9. For the lyase from M. vuglaris, the pI was around pH 4.6 and the pI for the lyase from M. costata was around 5.0. These values were obtained by isoelectric focusing on a gel with a pH gradient from 3 to 9.

The pI values deduced from the amino acid compositions are:

The lyase from the fungal infected G. lemaneiformis: 4.58 and for the lyase from M. costata: 6.30.

7.8. Immunological test of the lyase by Western blotting.

The results showed that the antibodies to the algal lyase could recognize the fungal lyase both in cell-free extracts and in purified form, as revealed by Western blottings. The antibodies to the algal lyase purified form the algae collected from China also recognized the lyase from the algae collected from Sant Cruz, Califonia.

80

GL sources	Reactivity with the antibodies against the GL from the fungal infected G. lemaneiformis
Fungal infected G. lemaneiformis	Strong
G. lemaneiformis from Califonia both form of GL	Strong
M. costata	medium
M. vulgaris	medium

7.9. Reversible and Irreversible Inhibitors of the fungal lyase

9.1. The reversible inhibitors, Glucose and Maltose.

At a substrate concentration of 10mg/ml, the activity for the M. costata lyase decreased by 19.3 % in the presence of 0.1 M glucose when amylopectin was used as substrate; the activity was not affected when glycogen was used as substrate. In the presence of 0.1 M of maltose the activity decreased by 48.8 % and 73.4%, respectively for glycogen and amylopectin.

Substrates Concentrations	Inhibitors Glucose	Maltose
Amylopectin 1% (2%)	19.3%(7%)	73.4% (67.2%)
Glycogen 1% (2%)	0.000 (-)	48.8% (49.7%)

It seems that the inhibition by 0.1 M glucose is competitive as increasing the substrate from 1% to 2% decreased the inhibition from 19.3 to 7%, whereas the inhibition by 0.1 M maltose is non-competitive as the increase of substrate did not significantly affect the inhibition degree.

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For the *M. vulgaris* lyase, 0.1 M glucose and maltose did also inhibit the reaction when either amylopectin or glycogen was used as substrate.

Substrates	Glucose	Maltose
Amylopectin (1%)	28%	80%
Glycogen (1%)	5%	57%

9.2. The reversible inhibitor deoxyjirimycin

At a final substrate concentration of 2%, the activity was decreased to 10.4% for the algal lyase and the *M.costata* lyase in the presence of 25 μM of deoxyjirimycin, using amylopectin as substrate. At 100 μM, the activity of both lyases was completely lost.

9.3. Irreversible Inhibitor: PCMB

Under the same assay conditions and in the presence of 2 mM PCMB, the activity decreased by 60% for the *M. costata* lyase and 98 % for the lyase from the fungal infected red algae. This means that the fungal lyase was much less sensitive to heavy metal inhibition.

7.10. Examples of laboratory scale production of AF

10.1. Production of AF using dextrin as substrate

The reactor contained 1000 g dextrins (obtained by treatment of starch with Termamyl to a final reducing matter of 10 %) in a final volume of 4.6 liter (HOAC-NaOAC, pH 3.9, containing 5 mM Na$_2$-EDTA). The reaction was initiated by adding 3 mg lyase purified from fungal infected algae. The reaction was performed at room temperature. At day 19, another batch of lyase (4 mg) was added.

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Reaction time (days)						
0	1	7	13	19	24	31

AF produced (grams)						
0	18	116	195	264	500	668

10.2. Using ^{14}C-Starch for the production of ^{14}C-AF

The uniformly labelled ^{14}C-starch (340 μCi obtained from Sigma) was vaccum-dried to remove the ethanol it contained and then dissolved in 2 ml water. The reaction was initiated by adding 20 μl lyase purified from the fungal infected algae and 20 μl pullulanase (MegaZyme Ltd.) The reaction was performed overnight at 30 °C. At the end of the reaction, the reaction mixture was filtered using a filter with a molecular mass cut off of 10,000 to remove the enzymes and unreacted starch molecules.

The filtrate was applied on a Ca$_2$ carbohydrate column (Chrompack) using a Waters HPLC. Water was used as eluent. The flow rate was 0.5 ml/min. AF was efficiently separated from glucose and maltosaccharides. The pooled AF fractions were freeze-dried and totally 140 μCi ^{14}C-AF was obtained.

These findings relate to an even further aspect of the present invention, namely the use of a reagent that can increase the hydrophobicity of the reaction medium (preferably an alcohol) to increase the stability and activity of the lyase according to the present invention. This increased stability leads to a increased AF yield.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: DANISCO A/S
        (B) STREET: LANGEBROGADE 1
        (C) CITY: COPENHAGEN
        (D) STATE: COPENHAGEN K
        (E) COUNTRY: DENMARK
        (F) POSTAL CODE (ZIP): DK-1001

    (ii) TITLE OF INVENTION: USE OF AN ENZYME

    (iii) NUMBER OF SEQUENCES: 39

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

    (v) CURRENT APPLICATION DATA:
        APPLICATION NUMBER: WO PCT/EP94/03397

(2) INFORMATION FOR SEQ ID NO: 1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1088 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Phe Ser Thr Leu Ala Phe Val Ala Pro Ser Ala Leu Gly Ala Ser
1   5   10   15

Thr Phe Val Gly Ala Glu Val Arg Ser Asn Val Arg Ile His Ser Ala
            20   25   30

Phe Pro Ala Val His Thr Ala Thr Arg Lys Thr Asn Arg Leu Asn Val
        35   40   45

Ser Met Thr Ala Leu Ser Asp Lys Gln Thr Ala Thr Ala Gly Ser Thr
    50   55   60

Asp Asn Pro Asp Gly Ile Asp Tyr Lys Thr Tyr Asp Tyr Val Gly Val
65   70   75   80

Trp Gly Phe Ser Pro Leu Ser Asn Thr Asn Trp Phe Ala Ala Gly Ser
            85   90   95

84

```
Ser Thr Pro Gly Gly Ile Thr Asp Trp Thr Ala Thr Met Asn Val Asn
            100             105                 110

Phe Asp Arg Ile Asp Asn Pro Ser Ile Thr Val Gln His Pro Val Gln
            115             120                 125

Val Gln Val Thr Ser Tyr Asn Asn Asn Ser Tyr Arg Val Arg Phe Asn
    130             135                 140

Pro Asp Gly Pro Ile Arg Asp Val Thr Arg Gly Pro Ile Leu Lys Gln
145             150                 155                 160

Gln Leu Asp Trp Ile Arg Thr Gln Glu Leu Ser Glu Gly Cys Asp Pro
                165                 170                 175

Gly Met Thr Phe Thr Ser Glu Gly Phe Leu Thr Phe Glu Thr Lys Asp
            180             185                 190

Leu Ser Val Ile Ile Tyr Gly Asn Phe Lys Thr Arg Val Thr Arg Lys
            195             200                 205

Ser Asp Gly Lys Val Ile Met Glu Asn Asp Glu Val Gly Thr Ala Ser
    210             215                 220

Ser Gly Asn Lys Cys Arg Gly Leu Met Phe Val Asp Arg Leu Tyr Gly
225             230             235                 240

Asn Ala Ile Ala Ser Val Asn Lys Asn Phe Arg Asn Asp Ala Val Lys
            245             250                 255

Gln Glu Gly Phe Tyr Gly Ala Gly Glu Val Asn Cys Lys Tyr Gln Asp
            260             265                 270

Thr Tyr Ile Leu Glu Arg Thr Gly Ile Ala Met Thr Asn Tyr Asn Tyr
    275             280                 285

Asp Asn Leu Asn Tyr Asn Gln Trp Asp Leu Arg Pro Pro His His Asp
    290             295                 300

Gly Ala Leu Asn Pro Asp Tyr Tyr Ile Pro Met Tyr Tyr Ala Ala Pro
305             310                 315                 320

Trp Leu Ile Val Asn Gly Cys Ala Gly Thr Ser Glu Gln Tyr Ser Tyr
            325             330                 335

Gly Trp Phe Met Asp Asn Val Ser Gln Ser Tyr Met Asn Thr Gly Asp
            340             345                 350

Thr Thr Trp Asn Ser Gly Gln Glu Asp Leu Ala Tyr Met Gly Ala Gln
        355             360                 365

Tyr Gly Pro Phe Asp Gln His Phe Val Tyr Gly Ala Gly Gly Gly Met
    370             375                 380

Glu Cys Val Val Thr Ala Phe Ser Leu Leu Gln Gly Lys Glu Phe Glu
385             390                 395                 400
```

85

Asn Gln Val Leu Asn Lys Arg Ser Val Met Pro Pro Lys Tyr Val Phe
            405   410   415

Gly Phe Phe Gln Gly Val Phe Gly Thr Ser Ser Leu Leu Arg Ala His
            420   425   430

Met Pro Ala Gly Glu Asn Asn Ile Ser Val Glu Glu Ile Val Glu Gly
        435   440   445

Tyr Gln Asn Asn Asn Phe Pro Phe Glu Gly Leu Ala Val Asp Val Asp
    450   455   460

Met Gln Asp Asn Leu Arg Val Phe Thr Thr Lys Gly Glu Phe Trp Thr
465   470   475   480

Ala Asn Arg Val Gly Thr Gly Gly Asp Pro Asn Asn Arg Ser Val Phe
            485   490   495

Glu Trp Ala His Asp Lys Gly Leu Val Cys Gln Thr Asn Ile Thr Cys
            500   505   510

Phe Leu Arg Asn Asp Asn Glu Gly Gln Asp Tyr Glu Val Asn Gln Thr
        515   520   525

Leu Arg Glu Arg Gln Leu Tyr Thr Lys Asn Asp Ser Leu Thr Gly Thr
    530   535   540

Asp Phe Gly Met Thr Asp Asp Gly Pro Ser Asp Ala Tyr Ile Gly His
545   550   555   560

Leu Asp Tyr Gly Gly Gly Val Glu Cys Asp Ala Leu Phe Pro Asp Trp
            565   570   575

Gly Arg Pro Asp Val Ala Glu Trp Trp Gly Asn Asn Tyr Lys Lys Leu
        580   585   590

Phe Ser Ile Gly Leu Asp Phe Val Trp Gln Asp Met Thr Val Pro Ala
        595   600   605

Met Met Pro His Lys Ile Gly Asp Asp Ile Asn Val Lys Pro Asp Gly
    610   615   620

Asn Trp Pro Asn Ala Asp Asp Pro Ser Asn Gly Gln Tyr Asn Trp Lys
625   630   635   640

Thr Tyr His Pro Gln Val Leu Val Thr Asp Met Arg Tyr Glu Asn His
            645   650   655

Gly Arg Glu Pro Met Val Thr Gln Arg Asn Ile His Ala Tyr Thr Leu
        660   665   670

Cys Glu Ser Thr Arg Lys Glu Gly Ile Val Glu Asn Ala Asp Thr Leu
        675   680   685

Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser Arg Gly Gly Tyr Ile Gly
    690   695   700

86

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Asn Gln His Phe Gly Gly Met Trp Val Gly Asp Asn Ser Thr Thr Ser
705             710         715             720         -

Asn Tyr Ile Gln Met Met Ile Ala Asn Asn Ile Asn Met Asn Met Ser
            725             730             735

Cys Leu Pro Leu Val Gly Ser Asp Ile Gly Gly Phe Thr Ser Tyr Asp
            740             745             750

Asn Glu Asn Gln Arg Thr Pro Cys Thr Gly Asp Leu Met Val Arg Tyr
        755             760             765

Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His Tyr Asp Arg
    770             775             780

Trp Ile Glu Ser Lys Asp His Gly Lys Asp Tyr Gln Glu Leu Tyr Met
785             790             795             800

Tyr Pro Asn Glu Met Asp Thr Leu Arg Lys Phe Val Glu Phe Arg Tyr
            805             810             815

Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala Phe
        820             825             830

Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asn Asn Asp Ser Asn
        835             840             845

Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly Gly His Asp Gly
    850             855             860

Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu Arg
865             870             875             880

Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro Asp
            885             890             895

Phe Asp Thr Lys Pro Leu Glu Gly Ala Met Asn Gly Gly Asp Arg Ile
        900             905             910

Tyr Asn Tyr Pro Val Pro Gln Ser Glu Ser Pro Ile Phe Val Arg Glu
        915             920             925

Gly Ala Ile Leu Pro Thr Arg Tyr Thr Leu Asn Gly Glu Asn Lys Ser
    930             935             940

Leu Asn Thr Tyr Thr Asp Glu Asp Pro Leu Val Phe Glu Val Phe Pro
945             950             955             960

Leu Gly Asn Asn Arg Ala Asp Gly Met Cys Tyr Leu Asp Asp Gly Gly
            965             970             975

Val Thr Thr Asn Ala Glu Asp Asn Gly Lys Phe Ser Val Val Lys Val
        980             985             990

Ala Ala Glu Gln Asp Gly Gly Thr Glu Thr Ile Thr Phe Thr Asn Asp
        995             1000            1005
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SUBSTITUTE SHEET (RULE 26)

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Cys Tyr Glu Tyr Val Phe Gly Gly Pro Phe Tyr Val Arg Val Arg Gly
    1010            1015            1020

Ala Gln Ser Pro Ser Asn Ile His Val Ser Ser Gly Ala Gly Ser Gln
1025            1030            1035            1040

Asp Met Lys Val Ser Ser Ala Thr Ser Arg Ala Ala Leu Phe Asn Asp
            1045            1050            1055

Gly Glu Asn Gly Asp Phe Trp Val Asp Gln Glu Thr Asp Ser Leu Trp
            1060            1065            1070

Leu Lys Leu Pro Asn Val Val Leu Pro Asp Ala Val Ile Thr Ile Thr
        1075            1080            1085
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(2) INFORMATION FOR SEQ ID NO: 2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1091 amino acids
        (B) TYPE: amino acid ·
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Tyr Pro Thr Leu Thr Phe Val Ala Pro Ser Ala Leu Gly Ala Arg
1           5           10          15

Thr Phe Thr Cys Val Gly Ile Phe Arg Ser His Ile Leu Ile His Ser
        20          25          30

Val Val Pro Ala Val Arg Leu Ala Val Arg Lys Ser Asn Arg Leu Asn
    35          40          45

Val Ser Met Ser Ala Leu Phe Asp Lys Pro Thr Ala Val Thr Gly Gly
    50          55          60

Lys Asp Asn Pro Asp Asn Ile Asn Tyr Thr Thr Tyr Asp Tyr Val Pro
65          70          75          80

Val Trp Arg Phe Asp Pro Leu Ser Asn Thr Asn Trp Phe Ala Ala Gly
        85          90          95

Ser Ser Thr Pro Gly Asp Ile Asp Asp Trp Thr Ala Thr Met Asn Val
        100         105         110

Asn Phe Asp Arg Ile Asp Asn Pro Ser Phe Thr Leu Glu Lys Pro Val
        115         120         125

Gln Val Gln Val Thr Ser Tyr Lys Asn Asn Cys Phe Arg Val Arg Phe
    130         135         140
```

88

Asn Pro Asp Gly Pro Ile Arg Asp Val Asp Arg Gly Pro Ile Leu Gln
145   150   155   160

Gln Gln Leu Asn Trp Ile Arg Lys Gln Glu Gln Ser Lys Gly Phe Asp
                165   170   175

Pro Lys Met Gly Phe Thr Lys Glu Gly Phe Leu Lys Phe Glu Thr Lys
            180   185   190

Asp Leu Asn Val Ile Ile Tyr Gly Asn Phe Lys Thr Arg Val Thr Arg
        195   200   205

Lys Arg Asp Gly Lys Gly Ile Met Glu Asn Asn Glu Val Pro Ala Gly
    210   215   220

Ser Leu Gly Asn Lys Cys Arg Gly Leu Met Phe Val Asp Arg Leu Tyr
225   230   235   240

Gly Thr Ala Ile Ala Ser Val Asn Glu Asn Tyr Arg Asn Asp Pro Asp
            245   250   255

Arg Lys Glu Gly Phe Tyr Gly Ala Gly Glu Val Asn Cys Glu Phe Trp
            260   265   270

Asp Ser Glu Gln Asn Arg Asn Lys Tyr Ile Leu Glu Arg Thr Gly Ile
        275   280   285

Ala Met Thr Asn Tyr Asn Tyr Asp Asn Tyr Asn Tyr Asn Gln Ser Asp
    290   295   300

Leu Ile Ala Pro Gly Tyr Pro Ser Asp Pro Asn Phe Tyr Ile Pro Met
305   310   315   320

Tyr Phe Ala Ala Pro Trp Val Val Val Lys Gly Cys Ser Gly Asn Ser
            325   330   335

Asp Glu Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val Ser Gln Thr
        340   345   350

Tyr Met Asn Thr Gly Gly Thr Ser Trp Asn Cys Gly Glu Glu Asn Leu
    355   360   365

Ala Tyr Met Gly Ala Gln Cys Gly Pro Phe Asp Gln His Phe Val Tyr
    370   375   380

Gly Asp Gly Asp Gly Leu Glu Asp Val Val Gln Ala Phe Ser Leu Leu
385   390   395   400

Gln Gly Lys Glu Phe Glu Asn Gln Val Leu Asn Lys Arg Ala Val Met
            405   410   415

Pro Pro Lys Tyr Val Phe Gly Tyr Phe Gln Gly Val Phe Gly Ile Ala
            420   425   430

Ser Leu Leu Arg Glu Gln Arg Pro Glu Gly Gly Asn Asn Ile Ser Val
    435   440   445

89

```
Gln Glu Ile Val Glu Gly Tyr Gln Ser Asn Asn Phe Pro Leu Glu Gly
    450             455             460

Leu Ala Val Asp Val Asp Met Gln Gln Asp Leu Arg Val Phe Thr Thr
465             470             475             480

Lys Ile Glu Phe Trp Thr Ala Asn Lys Val Gly Thr Gly Gly Asp Ser
            485             490             495

Asn Asn Lys Ser Val Phe Glu Trp Ala His Asp Lys Gly Leu Val Cys
            500             505             510

Gln Thr Asn Val Thr Cys Phe Leu Arg Asn Asp Asn Gly Gly Ala Asp
        515             520             525

Tyr Glu Val Asn Gln Thr Leu Arg Glu Lys Gly Leu Tyr Thr Lys Asn
    530             535             540

Asp Ser Leu Thr Asn Thr Asn Phe Gly Thr Thr Asn Asp Gly Pro Ser
545             550             555             560

Asp Ala Tyr Ile Gly His Leu Asp Tyr Gly Gly Gly Gly Asn Cys Asp
            565             570             575

Ala Leu Phe Pro Asp Trp Gly Arg Pro Gly Val Ala Glu Trp Trp Gly
        580             585             590

Asp Asn Tyr Ser Lys Leu Phe Lys Ile Gly Leu Asp Phe Val Trp Gln
    595             600             605

Asp Met Thr Val Pro Ala Met Met Pro His Lys Val Gly Asp Ala Val
    610             615             620

Asp Thr Arg Ser Pro Tyr Gly Trp Pro Asn Glu Asn Asp Pro Ser Asn
625             630             635             640

Gly Arg Tyr Asn Trp Lys Ser Tyr His Pro Gln Val Leu Val Thr Asp
            645             650             655

Met Arg Tyr Glu Asn His Gly Arg Glu Pro Met Phe Thr Gln Arg Asn
        660             665             670

Met His Ala Tyr Thr Leu Cys Glu Ser Thr Arg Lys Glu Gly Ile Val
        675             680             685

Ala Asn Ala Asp Thr Leu Thr Lys Phe Arg Arg Ser Tyr Ile Ile Ser
    690             695             700

Arg Gly Gly Tyr Ile Gly Asn Gln His Phe Gly Gly Met Trp Val Gly
705             710             715             720

Asp Asn Ser Ser Ser Gln Arg Tyr Leu Gln Met Met Ile Ala Asn Ile
            725             730             735

Val Asn Met Asn Met Ser Cys Leu Pro Leu Val Gly Ser Asp Ile Gly
        740             745             750
```

90

```
Gly Phe Thr Ser Tyr Asp Gly Arg Asn Val Cys Pro Gly Asp Leu Met
        755             760             765

Val Arg Phe Val Gln Ala Gly Cys Leu Leu Pro Trp Phe Arg Asn His
        770             775             780

Tyr Gly Arg Leu Val Glu Gly Lys Gln Glu Gly Lys Tyr Tyr Gln Glu
785             790             795             800

Leu Tyr Met Tyr Lys Asp Glu Met Ala Thr Leu Arg Lys Phe Ile Glu
            805             810             815

Phe Arg Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn
        820             825             830

Ala Ala Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn
        835             840             845

Asp Arg Asn Val Arg Gly Ala Gln Asp Asp His Phe Leu Leu Gly Gly
    850             855             860

His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Thr
865             870             875             880

Thr Ser Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys Phe
            885             890             895

Gly Pro Asp Tyr Asp Thr Lys Arg Leu Asp Ser Ala Leu Asp Gly Gly
        900             905             910

Gln Met Ile Lys Asn Tyr Ser Val Pro Gln Ser Asp Ser Pro Ile Phe
        915             920             925

Val Arg Glu Gly Ala Ile Leu Pro Thr Arg Tyr Thr Leu Asp Gly Ser
    930             935             940

Asn Lys Ser Met Asn Thr Tyr Thr Asp Lys Asp Pro Leu Val Phe Glu
945             950             955             960

Val Phe Pro Leu Gly Asn Asn Arg Ala Asp Gly Met Cys Tyr Leu Asp
            965             970             975

Asp Gly Gly Ile Thr Thr Asp Ala Glu Asp His Gly Lys Phe Ser Val
            980             985             990

Ile Asn Val Glu Ala Leu Arg Lys Gly Val Thr Thr Thr Ile Lys Phe
        995             1000            1005

Ala Tyr Asp Thr Tyr Gln Tyr Val Phe Asp Gly Pro Phe Tyr Val Arg
    1010            1015            1020

Ile Arg Asn Leu Thr Thr Ala Ser Lys Ile Asn Val Ser Ser Gly Ala
1025            1030            1035            1040

Gly Glu Glu Asp Met Thr Pro Thr Ser Ala Asn Ser Arg Ala Ala Leu
            1045            1050            1055
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91

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Phe Ser Asp Gly Gly Val Gly Glu Tyr Trp Ala Asp Asn Asp Thr Ser
        1060            1065            1070

Ser Leu Trp Met Lys Leu Pro Asn Leu Val Leu Gln Asp Ala Val Ile
        1075            1080            1085

Thr Ile Thr
    1090
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(2) INFORMATION FOR SEQ ID NO: 3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3267 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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ATGTTTTCAA CCCTTGCGTT TGTCGCACCT AGTGCGCTGG GAGCCAGTAC CTTCGTAGGG      60

GCGGAGGTCA GGTCAAATGT TCGTATCCAT TCCGCTTTTC CAGCTGTGCA CACAGCTACT     120

CGCAAAACCA ATCGCCTCAA TGTATCCATG ACCGCATTGT CCGACAAACA AACGGCTACT     180

GCGGGTAGTA CAGACAATCC GGACGGTATC GACTACAAGA CCTACGATTA CGTCGGAGTA     240

TGGGGTTTCA GCCCCCTCTC CAACACGAAC TGGTTTGCTG CCGGCTCTTC TACCCCGGGT     300

GGCATCACTG ATTGGACGGC TACAATGAAT GTCAACTTCG ACCGTATCGA CAATCCGTCC     360

ATCACTGTCC AGCATCCCGT TCAGGTTCAG GTCACGTCAT ACAACAACAA CAGCTACAGG     420

GTTCGCTTCA ACCCTGATGG CCCTATTCGT GATGTGACTC GTGGGCCTAT CCTCAAGCAG     480

CAACTAGATT GGATTCGAAC GCAGGAGCTG TCAGAGGGAT GTGATCCCGG AATGACTTTC     540

ACATCAGAAG GTTTCTTGAC TTTTGAGACC AAGGATCTAA GCGTCATCAT CTACGGAAAT     600

TTCAAGACCA GAGTTACGAG AAAGTCTGAC GGCAAGGTCA TCATGGAAAA TGATGAAGTT     660

GGAACTGCAT CGTCCGGGAA CAAGTGCCGG GGATTGATGT TCGTTGATAG ATTATACGGT     720

AACGCTATCG CTTCCGTCAA CAAGAACTTC CGCAACGACG CGGTCAAGCA GGAGGGATTC     780

TATGGTGCAG GTGAAGTCAA CTGTAAGTAC CAGGACACCT ACATCTTAGA ACGCACTGGA     840

ATCGCCATGA CAAATTACAA CTACGATAAC TTGAACTATA ACCAGTGGGA CCTTAGACCT     900

CCGCATCATG ATGGTGCCCT CAACCCAGAC TATTATATTC CAATGTACTA CGCAGCACCT     960

TGGTTGATCG TTAATGGATG CGCCGGTACT TCGGAGCAGT ACTCGTATGG ATGGTTCATG    1020
```

92

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GACAATGTCT CTCAATCTTA CATGAATACT GGAGATACTA CCTGGAATTC TGGACAAGAG   1080
GACCTGGCAT ACATGGGCGC GCAGTATGGA CCATTTGACC AACATTTTGT TTACGGTGCT   1140
GGGGGTGGGA TGGAATGTGT GGTCACAGCG TTCTCTCTTC TACAAGGCAA GGAGTTCGAG   1200
AACCAAGTTC TCAACAAACG TTCAGTAATG CCTCCGAAAT ACGTCTTTGG TTTCTTCCAG   1260
GGTGTTTTCG GGACTTCTTC CTTGTTGAGA GCGCATATGC CAGCAGGTGA GAACAACATC   1320
TCAGTCGAAG AAATTGTAGA AGGTTATCAA AACAACAATT TCCCTTTCGA GGGGCTCGCT   1380
GTGGACGTGG ATATGCAAGA CAACTTGCGG GTGTTCACCA CGAAGGGCGA ATTTTGGACC   1440
GCAAACAGGG TGGGTACTGG CGGGGATCCA AACAACCGAT CGGTTTTTGA ATGGGCACAT   1500
GACAAAGGCC TTGTTTGTCA GACAAATATA ACTTGCTTCC TGAGGAATGA TAACGAGGGG   1560
CAAGACTACG AGGTCAATCA GACGTTAAGG GAGAGGCAGT TGTACACGAA GAACGACTCC   1620
CTGACGGGTA CGGATTTTGG AATGACCGAC GACGGCCCCA GCGATGCGTA CATCGGTCAT   1680
CTGGACTATG GGGGTGGAGT AGAATGTGAT GCACTTTTCC CAGACTGGGG ACGGCCTGAC   1740
GTGGCCGAAT GGTGGGGAAA TAACTATAAG AAACTGTTCA GCATTGGTCT CGACTTCGTC   1800
TGGCAAGACA TGACTGTTCC AGCAATGATG CCGCACAAAA TTGGCGATGA CATCAATGTG   1860
AAACCGGATG GGAATTGGCC GAATGCGGAC GATCCGTCCA ATGGACAATA CAACTGGAAG   1920
ACGTACCATC CCCAAGTGCT TGTAACTGAT ATGCGTTATG AGAATCATGG TCGGGAACCG   1980
ATGGTCACTC AACGCAACAT TCATGCGTAT ACACTGTGCG AGTCTACTAG GAAGGAAGGG   2040
ATCGTGGAAA ACGCAGACAC TCTAACGAAG TTCCGCCGTA GCTACATTAT CAGTCGTGGT   2100
GGTTACATTG GTAACCAGCA TTTCGGGGGT ATGTGGGTGG GAGACAACTC TACTACATCA   2160
AACTACATCC AAATGATGAT TGCCAACAAT ATTAACATGA ATATGTCTTG CTTGCCTCTC   2220
GTCGGCTCCG ACATTGGAGG ATTCACCTCA TACGACAATG AGAATCAGCG AACGCCGTGT   2280
ACCGGGGACT TGATGGTGAG GTATGTGCAG GCGGGCTGCC TGTTGCCGTG GTTCAGGAAC   2340
CACTATGATA GGTGGATCGA GTCCAAGGAC CACGGAAAGG ACTACCAGGA GCTGTACATG   2400
TATCCGAATG AAATGGATAC GTTGAGGAAG TTCGTTGAAT CCGTTATCG CTGGCAGGAA    2460
GTGTTGTACA CGGCCATGTA CCAGAATGCG GCTTTCGGAA AGCCGATTAT CAAGGCTGCT   2520
TCGATGTACA ATAACGACTC AAACGTTCGC AGGGCGCAGA ACGATCATTT CCTTCTTGGT   2580
GGACATGATG GATATCGCAT TCTGTGCGCG CCTGTTGTGT GGGAGAATTC GACCGAACGC   2640
GAATTGTACT TGCCCGTGCT GACCCAATGG TACAAATTCG GTCCCGACTT TGACACCAAG   2700
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CCTCTGGAAG GAGCGATGAA CGGAGGGGAC CGAATTTACA ACTACCCTGT ACCGCAAAGT    2760

GAATCACCAA TCTTCGTGAG AGAAGGTGCG ATTCTCCCTA CCCGCTACAC GTTGAACGGT    2820

GAAAACAAAT CATTGAACAC GTACACGGAC GAAGATCCGT TGGTGTTTGA AGTATTCCCC    2880

CTCGGAAACA ACCGTGCCGA CGGTATGTGT TATCTTGATG ATGGCGGTGT GACCACCAAT    2940

GCTGAAGACA ATGGCAAGTT CTCTGTCGTC AAGGTGGCAG CGGAGCAGGA TGGTGGTACG    3000

GAGACGATAA CGTTTACGAA TGATTGCTAT GAGTACGTTT TCGGTGGACC GTTCTACGTT    3060

CGAGTGCGCG GCGCTCAGTC GCCGTCGAAC ATCCACGTGT CTTCTGGAGC GGGTTCTCAG    3120

GACATGAAGG TGAGCTCTGC CACTTCCAGG GCTGCGCTGT TCAATGACGG GGAGAACGGT    3180

GATTTCTGGG TTGACCAGGA GACAGATTCT CTGTGGCTGA AGTTGCCCAA CGTTGTTCTC    3240

CCGGACGCTG TGATCACAAT TACCTAA                                        3267
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(2) INFORMATION FOR SEQ ID NO: 4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3276 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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ATGTATCCAA CCCTCACCTT CGTGGCGCCT AGTGCGCTAG GGGCCAGAAC TTTCACGTGT    60

GTGGGCATTT TTAGGTCACA CATTCTTATT CATTCGGTTG TTCCAGCGGT GCGTCTAGCT    120

GTGCGCAAAA GCAACCGCCT CAATGTATCC ATGTCCGCTT TGTTCGACAA ACCGACTGCT    180

GTTACTGGAG GGAAGGACAA CCCGGACAAT ATCAATTACA CCACTTATGA CTACGTCCCT    240

GTGTGGCGCT TCGACCCCCT CAGCAATACG AACTGGTTTG CTGCCGGATC TTCCACTCCC    300

GGCGATATTG ACGACTGGAC GGCGACAATG AATGTGAACT TCGACCGTAT CGACAATCCA    360

TCCTTCACTC TCGAGAAACC GGTTCAGGTT CAGGTCACGT CATACAAGAA CAATTGTTTC    420

AGGGTTCGCT TCAACCCTGA TGGTCCTATT CGCGATGTGG ATCGTGGGCC TATCCTCCAG    480

CAGCAACTAA ATTGGATCCG GAAGCAGGAG CAGTCGAAGG GGTTTGATCC TAAGATGGGC    540

TTCACAAAAG AAGGTTTCTT GAAATTTGAG ACCAAGGATC TGAACGTTAT CATATATGGC    600

AATTTTAAGA CTAGAGTTAC GAGGAAGAGG GATGGAAAAG GGATCATGGA GAATAATGAA    660
```

94

```
GTGCCGGCAG GATCGTTAGG GAACAAGTGC CGGGGATTGA TGTTTGTCGA CAGGTTGTAC    720

GGCACTGCCA TCGCTTCCGT TAATGAAAAT TACCGCAACG ATCCCGACAG GAAAGAGGGG    780

TTCTATGGTG CAGGAGAAGT AAACTGCGAG TTTTGGGACT CCGAACAAAA CAGGAACAAG    840

TACATCTTAG AACGAACTGG AATCGCCATG ACAAATTACA ATTATGACAA CTATAACTAC    900

AACCAGTCAG ATCTTATTGC TCCAGGATAT CCTTCCGACC CGAACTTCTA CATTCCCATG    960

TATTTTGCAG CACCTTGGGT AGTTGTTAAG GGATGCAGTG GCAACAGCGA TGAACAGTAC   1020

TCGTACGGAT GGTTTATGGA TAATGTCTCC CAAACTTACA TGAATACTGG TGGTACTTCC   1080

TGGAACTGTG GAGAGGAGAA CTTGGCATAC ATGGGAGCAC AGTGCGGTCC ATTTGACCAA   1140

CATTTTGTGT ATGGTGATGG AGATGGTCTT GAGGATGTTG TCCAAGCGTT CTCTCTTCTG   1200

CAAGGCAAAG AGTTTGAGAA CCAAGTTCTG AACAAACGTG CCGTAATGCC TCCGAAATAT   1260

GTGTTTGGTT ACTTTCAGGG AGTCTTTGGG ATTGCTTCCT TGTTGAGAGA GCAAAGACCA   1320

GAGGGTGGTA ATAACATCTC TGTTCAAGAG ATTGTCGAAG GTTACCAAAG CAATAACTTC   1380

CCTTTAGAGG GGTTAGCCGT AGATGTGGAT ATGCAACAAG ATTTGCGCGT GTTCACCACG   1440

AAGATTGAAT TTTGGACGGC AAATAAGGTA GGCACCGGGG GAGACTCGAA TAACAAGTCG   1500

GTGTTTGAAT GGGCACATGA CAAAGGCCTT GTATGTCAGA CGAATGTTAC TTGCTTCTTG   1560

AGAAACGACA ACGGCGGGGC AGATTACGAA GTCAATCAGA CATTGAGGGA GAAGGGTTTG   1620

TACACGAAGA ATGACTCACT GACGAACACT AACTTCGGAA CTACCAACGA CGGGCCGAGC   1680

GATGCGTACA TTGGACATCT GGACTATGGT GGCGGAGGGA ATTGTGATGC ACTTTTCCCA   1740

GACTGGGGTC GACCGGGTGT GGCTGAATGG TGGGGTGATA ACTACAGCAA GCTCTTCAAA   1800

ATTGGTCTGG ATTTCGTCTG GCAAGACATG ACAGTTCCAG CTATGATGCC ACACAAAGTT   1860

GGCGACGCAG TCGATACGAG ATCACCTTAC GGCTGGCCGA ATGAGAATGA TCCTTCGAAC   1920

GGACGATACA ATTGGAAATC TTACCATCCA CAAGTTCTCG TAACTGATAT GCGATATGAG   1980

AATCATGGAA GGGAACCGAT GTTCACTCAA CGCAATATGC ATGCGTACAC ACTCTGTGAA   2040

TCTACGAGGA AGGAAGGGAT TGTTGCAAAT GCAGACACTC TAACGAAGTT CCGCCGCAGT   2100

TATATTATCA GTCGTGGAGG TTACATTGGC AACCAGCATT TTGGAGGAAT GTGGGTTGGA   2160

GACAACTCTT CCTCCCAAAG ATACCTCCAA ATGATGATCG CGAACATCGT CAACATGAAC   2220

ATGTCTTGCC TTCCACTAGT TGGGTCCGAC ATTGGAGGTT TTACTTCGTA TGATGGACGA   2280

AACGTGTGTC CCGGGGATCT AATGGTAAGA TTCGTGCAGG CGGGTTGCTT ACTACCGTGG   2340
```

95

```
TTCAGAAACC ACTATGGTAG GTTGGTCGAG GGCAAGCAAG AGGGAAAATA CTATCAAGAA    2400

CTGTACATGT ACAAGGACGA GATGGCTACA TTGAGAAAAT TCATTGAATT CCGTTACCGC    2460

TGGCAGGAGG TGTTGTACAC TGCTATGTAC CAGAATGCGG CTTTCGGGAA ACCGATTATC    2520

AAGGCAGCTT CCATGTACGA CAACGACAGA AACGTTCGCG GCGCACAGGA TGACCACTTC    2580

CTTCTCGGCG GACACGATGG ATATCGTATT TTGTGTGCAC CTGTTGTGTG GGAGAATACA    2640

ACCAGTCGCG ATCTGTACTT GCCTGTGCTG ACCAAATGGT ACAAATTCGG CCCTGACTAT    2700

GACACCAAGC GCCTGGATTC TGCGTTGGAT GGAGGGCAGA TGATTAAGAA CTATTCTGTG    2760

CCACAAAGCG ACTCTCCGAT ATTTGTGAGG GAAGGAGCTA TTCTCCCTAC CCGCTACACG    2820

TTGGACGGTT CGAACAAGTC AATGAACACG TACACAGACA AAGACCCGTT GGTGTTTGAG    2880

GTATTCCCTC TTGGAAACAA CCGTGCCGAC GGTATGTGTT ATCTTGATGA TGGCGGTATT    2940

ACTACAGATG CTGAGGACCA TGGCAAATTC TCTGTTATCA ATGTCGAAGC CTTACGGAAA    3000

GGTGTTACGA CGACGATCAA GTTTGCGTAT GACACTTATC AATACGTATT TGATGGTCCA    3060

TTCTACGTTC GAATCCGTAA TCTTACGACT GCATCAAAAA TTAACGTGTC TTCTGGAGCG    3120

GGTGAAGAGG ACATGACACC GACCTCTGCG AACTCGAGGG CAGCTTTGTT CAGTGATGGA    3180

GGTGTTGGAG AATACTGGGC TGACAATGAT ACGTCTTCTC TGTGGATGAA GTTGCCAAAC    3240

CTGGTTCTGC AAGACGCTGT GATTACCATT ACGTAG                              3276
```

(2) INFORMATION FOR SEQ ID NO: 5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1066 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Met Ala Gly Phe Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
1               5                   10                  15

Tyr Ser Val Ala Leu Asp Trp Lys Gly Pro Gln Lys Ile Ile Gly Val
            20                  25                  30

Asp Thr Thr Pro Pro Lys Ser Thr Lys Phe Pro Lys Asn Trp His Gly
        35                  40                  45

Val Asn Leu Arg Phe Asp Asp Gly Thr Leu Gly Val Val Gln Phe Ile
    50                  55                  60
```

96

```
Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Gly Phe Lys Thr Ser
65              70              75              80

Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
            85              90              95

Ser Thr Leu Ser Asn Lys Leu Asp Thr Tyr Arg Gly Leu Thr Trp Glu
            100             105             110

Thr Lys Cys Glu Asp Ser Gly Asp Phe Phe Thr Phe Ser Ser Lys Val
        115             120             125

Thr Ala Val Glu Lys Ser Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
    130             135             140

Leu Arg Ile His Leu Trp Lys Ser Pro Phe Arg Ile Gln Val Val Arg
145             150             155             160

Thr Leu Thr Pro Leu Lys Asp Pro Tyr Pro Ile Pro Asn Val Ala Ala
            165             170             175

Ala Glu Ala Arg Val Ser Asp Lys Val Val Trp Gln Thr Ser Pro Lys
            180             185             190

Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
        195             200             205

Val Leu Asp Ile Val Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
    210             215             220

Glu Met Gly Gly Ile Gln Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
225             230             235             240

Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
            245             250             255

Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
            260             265             270

Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
    275             280             285

Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
    290             295             300

Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
305             310             315             320

Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
            325             330             335

Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
        340             345             350

Tyr Gly Tyr Gln Gln Glu Ser Asp Leu Tyr Ser Val Val Gln Gln Tyr
    355             360             365
```

97

```
Arg Asp Cys Lys Phe Pro Leu Asp Gly Ile His Val Asp Val Asp Val
    370             375             380

Gln Asp Gly Phe Arg Thr Phe Thr Thr Asn Pro His Thr Phe Pro Asn
385             390             395             400

Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Asn Gly Ile Lys Cys Ser
            405             410             415

Thr Asn Ile Thr Pro Val Ile Ser Ile Asn Asn Arg Glu Gly Gly Tyr
            420             425             430

Ser Thr Leu Leu Glu Gly Val Asp Lys Lys Tyr Phe Ile Met Asp Asp
    435             440             445

Arg Tyr Thr Glu Gly Thr Ser Gly Asn Ala Lys Asp Val Arg Tyr Met
    450             455             460

Tyr Tyr Gly Gly Gly Asn Lys Val Glu Val Asp Pro Asn Asp Val Asn
465             470             475             480

Gly Arg Pro Asp Phe Lys Asp Asn Tyr Asp Phe Pro Ala Asn Phe Asn
            485             490             495

Ser Lys Gln Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
            500             505             510

Gly Ser Ala Gly Phe Tyr Pro Asp Leu Asn Arg Lys Glu Val Arg Ile
    515             520             525

Trp Trp Gly Met Gln Tyr Lys Tyr Leu Phe Asp Met Gly Leu Glu Phe
    530             535             540

Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Thr Ser Tyr Gly Asp
545             550             555             560

Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ser Asp Ser Val Thr
            565             570             575

Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Thr Trp Ala Leu Tyr Ser
            580             585             590

Tyr Asn Leu His Lys Ala Thr Trp His Gly Leu Ser Arg Leu Glu Ser
    595             600             605

Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
    610             615             620

Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Asn Trp
625             630             635             640

Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
            645             650             655

Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Tyr Arg
            660             665             670
```

98

```
Asp Ala Asn Gly Val Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile
        675                 680                 . 685

Arg Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr
    690                 695             700

Val Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ser Tyr Pro Lys
705             710             715                     720

His Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys
                725             730             735

Ser Val Leu Glu Ile Cys Arg Tyr Tyr Val Glu Leu Arg Tyr Ser Leu
            740             745                 750

Ile Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met
        755             760                 765

Pro Ile Thr Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe
    770             775             780

Phe Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp
785             790             795                     800

Asp Ile Leu Val Ala Pro Ile Leu His Ser Arg Lys Glu Ile Pro Gly
            805             810             815

Glu Asn Arg Asp Val Tyr Leu Pro Leu Tyr His Thr Trp Tyr Pro Ser
            820             825             830

Asn Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val
            835             840             845

Glu Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu
    850             855             860

Asp Tyr Asn Leu Phe His Ser Val Val Pro Val Tyr Val Arg Glu Gly
865             870             875                     880

Ala Ile Ile Pro Gln Ile Glu Val Arg Gln Trp Thr Gly Gln Gly Gly
            885             890             895

Ala Asn Arg Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr
            900             905             910

Cys Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Glu Asp
        915             920             925

Leu Pro Gln Tyr Lys Glu Thr His Glu Gln Ser Lys Val Glu Gly Ala
    930             935             940

Glu Ile Ala Lys Gln Ile Gly Lys Lys Thr Gly Tyr Asn Ile Ser Gly
945             950             955                     960

Thr Asp Pro Glu Ala Lys Gly Tyr His Arg Lys Val Ala Val Thr Gln
            965             970             975
```

99

```
Thr Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu Pro Lys His Asn
         980         985              990                      ` `

Gly Tyr Asp Pro Ser Lys Glu Val Gly Asp Tyr Tyr Thr Ile Ile Leu
         995              1000             1005

Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp Val Ser Lys Thr
    1010             1015             1020

Thr Val Asn Val Glu Gly Gly Val Glu His Gln Val Tyr Lys Asn Ser
1025             1030             1035              1040

Asp Leu His Thr Val Val Ile Asp Val Lys Glu Val Ile Gly Thr Thr
             1045             1050             1055

Lys Ser Val Lys Ile Thr Cys Thr Ala Ala
         1060             1065
```

(2) INFORMATION FOR SEQ ID NO: 6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1070 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Met Ala Gly Leu Ser Asp Pro Leu Asn Phe Cys Lys Ala Glu Asp Tyr
1             5             10            Lys     15

Tyr Ala Ala Ala Lys Gly Trp Ser Gly Pro Gln Lys Ile Ile Arg Tyr
         20              25              30

Asp Gln Thr Pro Pro Gln Gly Thr Lys Asp Pro Lys Ser Trp His Ala
         35              40              45

Val Asn Leu Pro Phe Asp Asp Gly Thr Met Cys Val Val Gln Phe Val
    50              55              60

Arg Pro Cys Val Trp Arg Val Arg Tyr Asp Pro Ser Val Lys Thr Ser
65              70              75                      80

Asp Glu Tyr Gly Asp Glu Asn Thr Arg Thr Ile Val Gln Asp Tyr Met
             85              90              95

Thr Thr Leu Val Gly Asn Leu Asp Ile Phe Arg Gly Leu Thr Trp Val
         100             105             110

Ser Thr Leu Glu Asp Ser Gly Glu Tyr Tyr Thr Phe Lys Ser Glu Val
         115             120             125
```

100

```
Thr Ala Val Asp Glu Thr Glu Arg Thr Arg Asn Lys Val Gly Asp Gly
    130               135                   140

Leu Lys Ile Tyr Leu Trp Lys Asn Pro Phe Arg Ile Gln Val Val Arg
    145           150               155               160

Leu Leu Thr Pro Leu Val Asp Pro Phe Pro Ile Pro Asn Val Ala Asn
            165               170               175

Ala Thr Ala Arg Val Ala Asp Lys Val Val Trp Gln Thr Ser Pro Lys
            180               185               190

Thr Phe Arg Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr
        195               200               205

Val Leu Asp Ile Ile Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly
    210               215               220

Glu Met Gly Gly Ile Glu Phe Met Lys Glu Pro Thr Phe Met Asn Tyr
225               230               235               240

Phe Asn Phe Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala
            245               250               255

Leu Asp Ser Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr Leu Asp
        260               265               270

Val Asn Ser Asn Pro Glu His Lys Asn Ile Thr Ala Thr Phe Ile Asp
        275               280               285

Asn Tyr Ser Gln Ile Ala Ile Asp Phe Gly Lys Thr Asn Ser Gly Tyr
    290               295               300

Ile Lys Leu Gly Thr Arg Tyr Gly Gly Ile Asp Cys Tyr Gly Ile Ser
305               310               315               320

Ala Asp Thr Val Pro Glu Ile Val Arg Leu Tyr Thr Gly Leu Val Gly
            325               330               335

Arg Ser Lys Leu Lys Pro Arg Tyr Ile Leu Gly Ala His Gln Ala Cys
            340               345               350

Tyr Gly Tyr Gln Gln Glu Ser Asp Leu His Ala Val Val Gln Gln Tyr
        355               360               365

Arg Asp Thr Lys Phe Pro Leu Asp Gly Leu His Val Asp Val Asp Phe
    370               375               380

Gln Asp Asn Phe Arg Thr Phe Thr Thr Asn Pro Ile Thr Phe Pro Asn
385               390               395               400

Pro Lys Glu Met Phe Thr Asn Leu Arg Asn Asn Gly Ile Lys Cys Ser
            405               410               415

Thr Asn Ile Thr Pro Val Ile Ser Ile Arg Asp Arg Pro Asn Gly Tyr
        420               425               430
```

101α

```
Ser Thr Leu Asn Glu Gly Tyr Asp Lys Lys Tyr Phe Ile Met Asp Asp
        435             440              445

Arg Tyr Thr Glu Gly Thr Ser Gly Asp Pro Gln Asn Val Arg Tyr Ser
    450             455             460

Phe Tyr Gly Gly Gly Asn Pro Val Glu Val Asn Pro Asn Asp Val Trp
465             470             475              480

Ala Arg Pro Asp Phe Gly Asp Asn Tyr Asp Phe Pro Thr Asn Phe Asn
            485             490              495

Cys Lys Asp Tyr Pro Tyr His Gly Gly Val Ser Tyr Gly Tyr Gly Asn
            500             505             510

Gly Thr Pro Gly Tyr Tyr Pro Asp Leu Asn Arg Glu Glu Val Arg Ile
        515             520             525

Trp Trp Gly Leu Gln Tyr Glu Tyr Leu Phe Asn Met Gly Leu Glu Phe
    530             535             540

Val Trp Gln Asp Met Thr Thr Pro Ala Ile His Ser Ser Tyr Gly Asp
545             550             555             560

Met Lys Gly Leu Pro Thr Arg Leu Leu Val Thr Ala Asp Ser Val Thr
            565             570             575

Asn Ala Ser Glu Lys Lys Leu Ala Ile Glu Ser Trp Ala Leu Tyr Ser
            580             585             590

Tyr Asn Leu His Lys Ala Thr Phe His Gly Leu Gly Arg Leu Glu Ser
        595             600             605

Arg Lys Asn Lys Arg Asn Phe Ile Leu Gly Arg Gly Ser Tyr Ala Gly
    610             615             620

Ala Tyr Arg Phe Ala Gly Leu Trp Thr Gly Asp Asn Ala Ser Thr Trp
625             630             635             640

Glu Phe Trp Lys Ile Ser Val Ser Gln Val Leu Ser Leu Gly Leu Asn
            645             650             655

Gly Val Cys Ile Ala Gly Ser Asp Thr Gly Gly Phe Glu Pro Ala Arg
        660             665             670

Thr Glu Ile Gly Glu Glu Lys Tyr Cys Ser Pro Glu Leu Leu Ile Arg
        675             680             685

Trp Tyr Thr Gly Ser Phe Leu Leu Pro Trp Leu Arg Asn His Tyr Val
    690             695             700

Lys Lys Asp Arg Lys Trp Phe Gln Glu Pro Tyr Ala Tyr Pro Lys His
705             710             715             720

Leu Glu Thr His Pro Glu Leu Ala Asp Gln Ala Trp Leu Tyr Lys Ser
            725             730             735
```

101 b

```
Val Leu Glu Ile Cys Arg Tyr Trp Val Glu Leu Arg Tyr Ser Leu Ile
            740               745               750

Gln Leu Leu Tyr Asp Cys Met Phe Gln Asn Val Val Asp Gly Met Pro
        755           760               765

Leu Ala Arg Ser Met Leu Leu Thr Asp Thr Glu Asp Thr Thr Phe Phe
    770           775               780

Asn Glu Ser Gln Lys Phe Leu Asp Asn Gln Tyr Met Ala Gly Asp Asp
785               790               795               800

Ile Leu Val Ala Pro Ile Leu His Ser Arg Asn Glu Val Pro Gly Glu
            805               810               815

Asn Arg Asp Val Tyr Leu Pro Leu Phe His Thr Trp Tyr Pro Ser Asn
            820               825               830

Leu Arg Pro Trp Asp Asp Gln Gly Val Ala Leu Gly Asn Pro Val Glu
        835               840               845

Gly Gly Ser Val Ile Asn Tyr Thr Ala Arg Ile Val Ala Pro Glu Asp
    850               855               860

Tyr Asn Leu Phe His Asn Val Val Pro Val Tyr Ile Arg Glu Gly Ala
865               870               875               880

Ile Ile Pro Gln Ile Gln Val Arg Gln Trp Ile Gly Glu Gly Gly Pro
            885               890               895

Asn Pro Ile Lys Phe Asn Ile Tyr Pro Gly Lys Asp Lys Glu Tyr Val
            900               905               910

Thr Tyr Leu Asp Asp Gly Val Ser Arg Asp Ser Ala Pro Asp Asp Leu
        915               920               925

Pro Gln Tyr Arg Glu Ala Tyr Glu Gln Ala Lys Val Glu Gly Lys Asp
    930               935               940

Val Gln Lys Gln Leu Ala Val Ile Gln Gly Asn Lys Thr Asn Asp Phe
945               950               955               960

Ser Ala Ser Gly Ile Asp Lys Glu Ala Lys Gly Tyr His Arg Lys Val
            965               970               975

Ser Ile Lys Gln Glu Ser Lys Asp Lys Thr Arg Thr Val Thr Ile Glu
        980               985               990

Pro Lys His Asn Gly Tyr Asp Pro Ser Lys Glu Val Gly Asn Tyr Tyr
    995               1000              1005

Thr Ile Ile Leu Trp Tyr Ala Pro Gly Phe Asp Gly Ser Ile Val Asp
    1010              1015              1020
```

101 c

```
Val Ser Gln Ala Thr Val Asn Ile Glu Gly Gly Val Glu Cys Glu Ile
1025                1030            1035                1040        .

Phe Lys Asn Thr Gly Leu His Thr Val Val Val Asn Val Lys Glu Val
              1045            1050            1055

Ile Gly Thr Thr Lys Ser Val Lys Ile Thr Cys Thr Thr Ala
          1060            1065            1070
```

(2) INFORMATION FOR SEQ ID NO: 7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3201 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
ATGGCAGGAT TTTCTGATCC TCTCAACTTT TGCAAAGCAG AAGACTACTA CAGTGTTGCG      60

CTAGACTGGA AGGGCCCTCA AAAAATCATT GGAGTAGACA CTACTCCTCC AAAGAGCACC     120

AAGTTCCCCA AAAACTGGCA TGGAGTGAAC TTGAGATTCG ATGATGGGAC TTTAGGTGTG     180

GTTCAGTTCA TTAGGCCGTG CGTTTGGAGG GTTAGATACG ACCCTGGTTT CAAGACCTCT     240

GACGAGTATG GTGATGAGAA TACGAGGACA ATTGTGCAAG ATTATATGAG TACTCTGAGT     300

AATAAATTGG ATACTTATAG AGGTCTTACG TGGGAAACCA AGTGTGAGGA TTCGGGAGAT     360

TTCTTTACCT TCTCATCCAA GGTCACCGCC GTTGAAAAAT CCGAGCGGAC CCGCAACAAG     420

GTCGGCGATG GCCTCAGAAT TCACCTATGG AAAAGCCCTT TCCGCATCCA AGTAGTGCGC     480

ACCTTGACCC CTTTGAAGGA TCCTTACCCC ATTCCAAATG TAGCCGCAGC CGAAGCCCGT     540

GTGTCCGACA AGGTCGTTTG GCAAACGTCT CCCAAGACAT TCAGAAAGAA CCTGCATCCG     600

CAACACAAGA TGCTAAAGGA TACAGTTCTT GACATTGTCA AACCTGGACA TGGCGAGTAT     660

GTGGGGTGGG GAGAGATGGG AGGTATCCAG TTTATGAAGG AGCCAACATT CATGAACTAT     720

TTTAACTTCG ACAATATGCA ATACCAGCAA GTCTATGCCC AAGGTGCTCT CGATTCTCGC     780

GAGCCACTGT ACCACTCGGA TCCCTTCTAT CTTGATGTGA ACTCCAACCC GGAGCACAAG     840

AATATCACGG CAACCTTTAT CGATAACTAC TCTCAAATTG CCATCGACTT TGGAAAGACC     900

AACTCAGGCT ACATCAAGCT GGGAACCAGG TATGGTGGTA TCGATTGTTA CGGTATCAGT     960

GCGGATACGG TCCCGGAAAT TGTACGACTT TATACAGGTC TTGTTGGACG TTCAAAGTTG    1020
```

102 a

```
AAGCCCAGAT ATATTCTCGG GGCCCATCAA GCCTGTTATG GATACCAACA GGAAAGTGAC   1080

TTGTATTCTG TGGTCCAGCA GTACCGTGAC TGTAAATTTC CACTTGACGG GATTCACGTC   1140

GATGTCGATG TTCAGGACGG CTTCAGAACT TTCACCACCA ACCCACACAC TTTCCCTAAC   1200

CCCAAAGAGA TGTTTACTAA CTTGAGGAAT AATGGAATCA AGTGCTCCAC CAATATCACT   1260

CCTGTTATCA GCATTAACAA CAGAGAGGGT GGATACAGTA CCCTCCTTGA GGGAGTTGAC   1320

AAAAAATACT TTATCATGGA CGACAGATAT ACCGAGGGAA CAAGTGGGAA TGCGAAGGAT   1380

GTTCGGTACA TGTACTACGG TGGTGGTAAT AAGGTTGAGG TCGATCCTAA TGATGTTAAT   1440

GGTCGGCCAG ACTTTAAAGA CAACTATGAC TTCCCCGCGA ACTTCAACAG CAAACAATAC   1500

CCCTATCATG GTGGTGTGAG CTACGGTTAT GGGAACGGTA GTGCAGGTTT TTACCCGGAC   1560

CTCAACAGAA AGGAGGTTCG TATCTGGTGG GGAATGCAGT ACAAGTATCT CTTCGATATG   1620

GGACTGGAAT TTGTGTGGCA AGACATGACT ACCCCAGCAA TCCACACATC ATATGGAGAC   1680

ATGAAAGGGT TGCCCACCCG TCTACTCGTC ACCTCAGACT CCGTCACCAA TGCCTCTGAG   1740

AAAAAGCTCG CAATTGAAAC TTGGGCTCTC TACTCCTACA ATCTCCACAA AGCAACTTGG   1800

CATGGTCTTA GTCGTCTCGA ATCTCGTAAG AACAAACGAA ACTTCATCCT CGGGCGTGGA   1860

AGTTATGCCG GAGCCTATCG TTTTGCTGGT CTCTGGACTG GGGATAATGC AAGTAACTGG   1920

GAATTCTGGA AGATATCGGT CTCTCAAGTT CTTTCTCTGG GCCTCAATGG TGTGTGCATC   1980

GCGGGGTCTG ATACGGGTGG TTTTGAACCC TACCGTGATG CAAATGGGGT CGAGGAGAAA   2040

TACTGTAGCC CAGAGCTACT CATCAGGTGG TATACTGGTT CATTCCTCTT GCCGTGGCTC   2100

AGGAACCATT ATGTCAAAAA GGACAGGAAA TGGTTCCAGG AACCATACTC GTACCCCAAG   2160

CATCTTGAAA CCCATCCAGA ACTCGCAGAC CAAGCATGGC TCTATAAATC CGTTTTGGAG   2220

ATCTGTAGGT ACTATGTGGA GCTTAGATAC TCCCTCATCC AACTACTTTA CGACTGCATG   2280

TTTCAAAACG TAGTCGACGG TATGCCAATC ACCAGATCTA TGCTCTTGAC CGATACTGAG   2340

GATACCACCT TCTTCAACGA GAGCCAAAAG TTCCTCGACA ACCAATATAT GGCTGGTGAC   2400

GACATTCTTG TTGCACCCAT CCTCCACAGT CGCAAAGAAA TTCCAGGCGA AAACAGAGAT   2460

GTCTATCTCC CTCTTTACCA CACCTGGTAC CCCTCAAATT TGAGACCATG GGACGATCAA   2520

GGAGTCGCTT TGGGGAATCC TGTCGAAGGT GGTAGTGTCA TCAATTATAC TGCTAGGATT   2580

GTTGCACCCG AGGATTATAA TCTCTTCCAC AGCGTGGTAC CAGTCTACGT TAGAGAGGGT   2640

GCCATCATCC CGCAAATCGA AGTACGCCAA TGGACTGGCC AGGGGGGAGC CAACCGCATC   2700
```

102 b

```
AAGTTCAACA TCTACCCTGG AAAGGATAAG GAGTACTGTA CCTATCTTGA TGATGGTGTT    2760

AGCCGTGATA GTGCGCCGGA AGACCTCCCA CAGTACAAAG AGACCCACGA ACAGTCGAAG    2820

GTTGAAGGCG CGGAAATCGC AAAGCAGATT GGAAAGAAGA CGGGTTACAA CATCTCAGGA    2880

ACCGACCCAG AAGCAAAGGG TTATCACCGC AAAGTTGCTG TCACACAAAC GTCAAAAGAC    2940

AAGACGCGTA CTGTCACTAT TGAGCCAAAA CACAATGGAT ACGACCCTTC CAAAGAGGTG    3000

GGTGATTATT ATACCATCAT TCTTTGGTAC GCACCAGGTT TCGATGGCAG CATCGTCGAT    3060

GTGAGCAAGA CGACTGTGAA TGTTGAGGGT GGGGTGGAGC ACCAAGTTTA TAAGAACTCC    3120

GATTTACATA CGGTTGTTAT CGACGTGAAG GAGGTGATCG GTACCACAAA GAGCGTCAAG    3180

ATCACATGTA CTGCCGCTTA A                                             3201
```

(2) INFORMATION FOR SEQ ID NO: 8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 3213 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
ATGGCAGGAT TATCCGACCC TCTCAATTTC TGCAAAGCAG AGGACTACTA CGCTGCTGCC     60

AAAGGCTGGA GTGGCCCTCA GAAGATCATT CGCTATGACC AGACCCCTCC TCAGGGTACA    120

AAAGATCCGA AAAGCTGGCA TGCGGTAAAC CTTCCTTTCG ATGACGGGAC TATGTGTGTA    180

GTGCAATTCG TCAGACCCTG TGTTTGGAGG GTTAGATATG ACCCCAGTGT CAAGACTTCT    240

GATGAGTACG GCGATGAGAA TACGAGGACT ATTGTACAAG ACTACATGAC TACTCTGGTT    300

GGAAACTTGG ACATTTTCAG AGGTCTTACG TGGGTTTCTA CGTTGGAGGA TTCGGGCGAG    360

TACTACACCT TCAAGTCCGA AGTCACTGCC GTGGACGAAA CCGAACGGAC TCGAAACAAG    420

GTCGGCGACG GCCTCAAGAT TTACCTATGG AAAAATCCCT TTCGCATCCA GGTAGTGCGT    480

CTCTTGACCC CCCTGGTGGA CCCTTTCCCC ATTCCCAACG TAGCCAATGC CACAGCCCGT    540

GTGGCCGACA AGGTTGTTTG GCAGACGTCC CCGAAGACGT TCAGGAAAAA CTTGCATCCG    600

CAGCATAAGA TGTTGAAGGA TACAGTTCTT GATATTATCA AGCCGGGGCA CGGAGAGTAT    660

GTGGGTTGGG GAGAGATGGG AGGCATCGAG TTTATGAAGG AGCCAACATT CATGAATTAT    720
```

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```
TTCAACTTTG ACAATATGCA ATATCAGCAG GTCTATGCAC AAGGCGCTCT TGATAGTCGT      780

GAGCCGTTGT ATCACTCTGA TCCCTTCTAT CTCGACGTGA ACTCCAACCC AGAGCACAAG      840

AACATTACGG CAACCTTTAT CGATAACTAC TCTCAGATTG CCATCGACTT TGGGAAGACC      900

AACTCAGGCT ACATCAAGCT GGGTACCAGG TATGGCGGTA TCGATTGTTA CGGTATCAGC      960

GCGGATACGG TCCCGGAGAT TGTGCGACTT TATACTGGAC TTGTTGGGCG TTCGAAGTTG     1020

AAGCCCAGGT ATATTCTCGG AGCCCACCAA GCTTGTTATG GATACCAGCA GGAAAGTGAC     1080

TTGCATGCTG TTGTTCAGCA GTACCGTGAC ACCAAGTTTC CGCTTGATGG GTTGCATGTC     1140

GATGTCGACT TTCAGGACAA TTTCAGAACG TTTACCACTA ACCCGATTAC GTTCCCTAAT     1200

CCCAAAGAAA TGTTTACCAA TCTAAGGAAC AATGGAATCA AGTGTTCCAC CAACATCACC     1260

CCTGTTATCA GTATCAGAGA TCGCCCGAAT GGGTACAGTA CCCTCAATGA GGGATATGAT     1320

AAAAAGTACT TCATCATGGA TGACAGATAT ACCGAGGGGA CAAGTGGGGA CCCGCAAAAT     1380

GTTCGATACT CTTTTTACGG CGGTGGGAAC CCGGTTGAGG TTAACCCTAA TGATGTTTGG     1440

GCTCGGCCAG ACTTTGGAGA CAATTATGAC TTCCCTACGA ACTTCAACTG CAAAGACTAC     1500

CCCTATCATG GTGGTGTGAG TTACGGATAT GGGAATGGCA CTCCAGGTTA CTACCCTGAC     1560

CTTAACAGAG AGGAGGTTCG TATCTGGTGG GGATTGCAGT ACGAGTATCT CTTCAATATG     1620

GGACTAGAGT TTGTATGGCA AGATATGACA ACCCCAGCGA TCCATTCATC ATATGGAGAC     1680

ATGAAAGGGT TGCCCACCCG TCTGCTCGTC ACCGCCGACT CAGTTACCAA TGCCTCTGAG     1740

AAAAAGCTCG CAATTGAAAG TTGGGCTCTT TACTCCTACA ACCTCCATAA AGCAACCTTC     1800

CACGGTCTTG GTCGTCTTGA GTCTCGTAAG AACAAACGTA ACTTCATCCT CGGACGTGGT     1860

AGTTACGCCG GTGCCTATCG TTTTGCTGGT CTCTGGACTG AGATAACGC AAGTACGTGG      1920

GAATTCTGGA AGATTTCGGT CTCCCAAGTT CTTTCTCTAG GTCTCAATGG TGTGTGTATA     1980

GCGGGGTCTG ATACGGGTGG TTTTGAGCCC GCACGTACTG AGATTGGGGA GGAGAAATAT    2040

TGCAGTCCGG AGCTACTCAT CAGGTGGTAT ACTGGATCAT TCCTTTTGCC ATGGCTTAGA    2100

AACCACTACG TCAAGAAGGA CAGGAAATGG TTCCAGGAAC CATACGCGTA CCCCAAGCAT    2160

CTTGAAACCC ATCCAGAGCT CGCAGATCAA GCATGGCTTT ACAAATCTGT TCTAGAAATT    2220

TGCAGATACT GGGTAGAGCT AAGATATTCC CTCATCCAGC TCCTTTACGA CTGCATGTTC    2280

CAAAACGTGG TCGATGGTAT GCCACTTGCC AGATCTATGC TCTTGACCGA TACTGAGGAT    2340

ACGACCTTCT TCAATGAGAG CCAAAAGTTC CTCGATAACC AATATATGGC TGGTGACGAC    2400
```

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```
ATCCTTGTAG CACCCATCCT CCACAGCCGT AACGAGGTTC CGGGAGAGAA CAGAGATGTC    2460

TATCTCCCTC TATTCCACAC CTGGTACCCC TCAAACTTGA GACCGTGGGA CGATCAGGGA    2520

GTCGCTTTAG GGAATCCTGT CGAAGGTGGC AGCGTTATCA ACTACACTGC CAGGATTGTT    2580

GCCCCAGAGG ATTATAATCT CTTCCACAAC GTGGTGCCGG TCTACATCAG AGAGGGTGCC    2640

ATCATTCCGC AAATTCAGGT ACGCCAGTGG ATTGGCGAAG GAGGGCCTAA TCCCATCAAG    2700

TTCAATATCT ACCCTGGAAA GGACAAGGAG TATGTGACGT ACCTTGATGA TGGTGTTAGC    2760

CGCGATAGTG CACCAGATGA CCTCCCGCAG TACCGCGAGG CCTATGAGCA AGCGAAGGTC    2820

GAAGGCAAAG ACGTCCAGAA GCAACTTGCG GTCATTCAAG GGAATAAGAC TAATGACTTC    2880

TCCGCCTCCG GGATTGATAA GGAGGCAAAG GGTTATCACC GCAAAGTTTC TATCAAACAG    2940

GAGTCAAAAG ACAAGACCCG TACTGTCACC ATTGAGCCAA AACACAACGG ATACGACCCC    3000

TCTAAGGAAG TTGGTAATTA TTATACCATC ATTCTTTGGT ACGCACCGGG CTTTGACGGC    3060

AGCATCGTCG ATGTGAGCCA GGCGACCGTG AACATCGAGG GCGGGGTGGA ATGCGAAATT    3120

TTCAAGAACA CCGGCTTGCA TACGGTTGTA GTCAACGTGA AAGAGGTGAT CGGTACCACA    3180

AAGTCCGTCA AGATCACTTG CACTACCGCT TAG                                 3213
```

(2) INFORMATION FOR SEQ ID NO: 9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 317 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Modified-site
        (B) LOCATION: 201
        (D) OTHER INFORMATION: /note= "X denotes a misc. amino
            acid"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

```
Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro Asp Leu
1               5               10              15

Ile Pro Pro Gly His Asp Ser Asp Pro Asp Tyr Tyr Ile Pro Met Tyr
            20              25              30

Phe Ala Ala Pro Trp Val Ile Ala His Gly Tyr Arg Gly Thr Ser Asp
        35              40              45
```

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```
Gln Tyr Ser Tyr Gly Trp Phe Leu Asp Asn Val Ser Gln Ser Tyr Thr
    50                  55              60
Asn Thr Gly Asp Asp Ala Trp Ala Gly Gln Lys Asp Leu Ala Tyr Met
65                  70              75                  80
Gly Ala Gln Cys Gly Pro Phe Asp Gln His Phe Val Tyr Glu Ala Gly
                85              90                  95
Asp Gly Leu Glu Asp Val Val Thr Ala Phe Ser Tyr Leu Gln Gly Lys
            100             105                 110
Glu Tyr Glu Asn Gln Gly Leu Asn Ile Arg Ser Ala Met Pro Pro Lys
        115                 120                 125
Tyr Val Phe Gly Phe Phe Gln Gly Val Phe Gly Ala Thr Ser Leu Leu
    130                 135                 140
Arg Asp Asn Leu Pro Ala Gly Glu Asn Asn Val Ser Leu Glu Glu Ile
145                 150                 155                 160
Val Glu Gly Tyr Gln Asn Gln Asn Val Pro Phe Glu Gly Leu Ala Val
            165                 170                 175
Asp Val Asp Met Gln Asp Asp Leu Arg Val Phe Thr Thr Arg Pro Ala
            180                 185                 190
Phe Trp Thr Ala Asn Lys Val Gly Xaa Gly Gly Asp Pro Asn Asn Lys
        195                 200                 205
Ser Val Phe Glu Trp Ala His Asp Arg Gly Leu Val Cys Gln Thr Asn
    210                 215                 220
Val Thr Cys Phe Leu Lys Asn Glu Lys Asn Pro Tyr Glu Val Asn Gln
225                 230                 235                 240
Ser Leu Arg Glu Lys Gln Leu Tyr Thr Lys Ser Asp Ser Leu Asp Asn
            245                 250                 255
Ile Asp Phe Gly Thr Thr Pro Asp Gly Pro Ser Asp Ala Tyr Ile Gly
            260                 265                 270
His Leu Asp Tyr Gly Gly Gly Val Glu Cys Asp Ala Leu Phe Pro Asp
        275                 280                 285
Trp Gly Arg Pro Asp Val Ala Gln Trp Trp Gly Asp Asn Tyr Lys Lys
    290                 295                 300
Leu Phe Ser Ile Gly Leu Asp Phe Val Trp Gln Asp Met
305                 310                 315
```

(2) INFORMATION FOR SEQ ID NO: 10:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 323 amino acids
       (B) TYPE: amino acid

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```
      (D) TOPOLOGY: linear

 (ii) MOLECULE TYPE: peptide


 (ix) FEATURE:
      (A) NAME/KEY: Modified-site
      (B) LOCATION: 272
      (D) OTHER INFORMATION: /note= "X is a misc. amino acid"

 (ix) FEATURE:
      (A) NAME/KEY: Modified-site
      (B) LOCATION: 273
      (D) OTHER INFORMATION: /note= "X is a misc. amino acids"

 (ix) FEATURE:
      (A) NAME/KEY: Modified-site
      (B) LOCATION: 274
      (D) OTHER INFORMATION: /note= "X is a misc. amino acid"


 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Thr Asn Tyr Asn Tyr Asp Asn Tyr Asn Tyr Asn Gln Ser Asp Leu
1               5                  10               15

Ile Ala Pro Gly Tyr Pro Ser Asp Pro Asn Phe Tyr Ile Pro Met Tyr
            20              25              30

Phe Ala Ala Pro Trp Val Val Val Lys Gly Cys Ser Gly Asn Ser Asp
        35              40              45

Glu Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val Ser Gln Thr Tyr
    50              55              60

Met Asn Thr Gly Gly Thr Ser Trp Asn Cys Gly Glu Glu Asn Leu Ala
65              70              75              80

Tyr Met Gly Ala Gln Cys Gly Pro Phe Asp Gln His Phe Val Tyr Gly
            85              90              95

Asp Gly Asp Gly Leu Glu Asp Val Val Gln Ala Phe Ser Leu Leu Gln
            100             105             110

Gly Lys Glu Phe Glu Asn Gln Val Leu Asn Lys Arg Ala Val Met Pro
        115             120             125

Pro Lys Tyr Val Phe Gly Tyr Phe Gln Gly Val Phe Gly Ile Ala Ser
    130             135             140

Leu Leu Arg Glu Gln Arg Pro Glu Gly Gly Asn Asn Ile Ser Val Ser
145             150             155             160

Glu Ile Val Glu Gly Tyr Gln Ser Asn Asn Phe Pro Leu Glu Gly Leu
            165             170             175
```

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```
Ala Val Asp Val Asp Met Gln Gln Asp Leu Arg Cys Ser Ser Pro Leu
             180             .        185            .     190
                                                                    _

Lys Ile Glu Phe Trp Thr Ala Asn Lys Val Gly Thr Gly Gly Asp Ser
         195             200             205

Asn Asn Lys Ser Val Phe Glu Trp Ala His Asp Lys Gly Leu Val Cys
     210             215             220

Gln Thr Asn Val Thr Cys Phe Leu Arg Asn Asp Asn Gly Gly Ala Asp
 225             230             235             240

Tyr Glu Val Asn Gln Thr Leu Arg Glu Lys Gly Leu Tyr Thr Lys Asn
             245             250             255 ·

Asp Ser Leu Thr Asn Thr Asn Phe Gly Thr Thr Asn Asp Gly Pro Xaa
         260             265             270

Xaa Xaa Tyr Ile Gly His Leu Asp Tyr Gly Gly Gly Gly Asn Cys Asp
         275             280             285

Ala Leu Phe Pro Asp Trp Gly Arg Pro Gly Val Ala Glu Trp Trp Gly
     290             295             300

Asp Asn Tyr Ser Lys Leu Phe Lys Ile Gly Leu Asp Phe Val Trp Gln
 305             310             315             320

Asp Met Thr
```

(2) INFORMATION FOR SEQ ID NO: 11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 202 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (ix) FEATURE:
        (A) NAME/KEY: Modified-site
        (B) LOCATION: 43
        (D) OTHER INFORMATION: /note= "X is a misc. amino acid"

    (ix) FEATURE:
        (A) NAME/KEY: Modified-site
        (B) LOCATION: 176
        (D) OTHER INFORMATION: /note= "X is a misc. amino acid"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

```
Met Thr Asn Tyr Asn Tyr Asp Asn Leu Asn Tyr Asn Gln Pro Asp Val
 1               5               10      _           15
```

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```
Val Pro Pro Gly Tyr His Asp His Pro Asn Tyr Tyr Ile Pro Met Tyr
        20              25                  30

Tyr Ala Ala Pro Trp Leu Val Val Gln Gly Xaa Ala Gly Thr Ser Lys
        35              40                  45

Gln Tyr Ser Tyr Gly Trp Phe Met Asp Asn Val Ser Gln Ser Tyr Met
    50              55              60

Asn Thr Gly Asp Thr Ala Trp Asn Cys Gly Gln Glu Asn Leu Ala Tyr
65              70              75                  80

Met Gly Ala Gln Tyr Gly Pro Phe Asp Gln His Phe Val Tyr Gly Asp
                85              90                  95

Gly Asp Gly Leu Glu Asp Val Val Lys Ala Phe Ser Phe Leu Gln Gly
            100             105             110

Lys Glu Phe Glu Asp Lys Lys Leu Asn Lys Arg Ser Val Met Pro Pro
            115             120             125

Lys Tyr Val Phe Gly Phe Phe Gln Gly Val Phe Gly Ala Leu Ser Leu
    130             135             140

Leu Lys Gln Asn Leu Pro Ala Gly Glu Asn Asn Ile Ser Val Gln Glu
145             150             155             160

Ile Val Glu Gly Tyr Gln Asp Asn Asp Tyr Pro Phe Glu Gly Leu Xaa
                165             170             175

Val Asp Val Asp Met Gln Asp Asp Leu Arg Val Phe Thr Thr Lys Pro
            180             185             190

Glu Tyr Trp Ser Ala Asn Met Val Gly Glu
    195             200
```

(2) INFORMATION FOR SEQ ID NO: 12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 953 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(573, "")
        (D) OTHER INFORMATION: /note= "g is a misc nucleic acid"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(601, "")
        (D) OTHER INFORMATION: /note= "g is a misc. nucleic acid"

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

```
ATGACAAACT ATAATTATGA CAATTTGAAC TACAATCAAC CGGACCTCAT CCCACCTGGC      60

CATGATTCAG ATCCTGACTA CTATATTCCG ATGTACTTTG CGGCACCATG GGTGATCGCA     120

CATGGATATC GTGGCACCAG CGACCAGTAC TCTTATGGAT GGTTTTTGGA CAATGTATCC     180

CAGTCCTACA CAAACACTGG CGATGATGCA TGGGCTGGTC AGAAGGATTT GGCGTACATG     240

GGGGCACAAT GTGGGCCTTT CGATCAACAT TTTGTGTATG AGGCTGGAGA TGGACTTGAA     300

GACGTTGTGA CCGCATTCTC TTATTTGCAA GGCAAGGAAT ATGAGAACCA GGGACTGAAT     360

ATACGTTCTG CAATGCCTCC GAAGTACGTT TTCGGATTTT TCCAAGGCGT ATTCGGAGCC     420

ACATCGCTGC TAAGGGACAA CTTACCTGCC GGCGAGAACA ACGTCTCTTT GGAAGAAATT     480

GTTGAAGGAT ATCAAAATCA GAACGTGCCA TTTGAAGGTC TTGCTGTGGA TGTTGATATG     540

CAAGATGACT TGAGAGTGTT CACTACGAGA CCGGCGTTTT GGACGGCAAA CAAGGTGGGG     600

GAAGGCGGTG ATCCAAACAA CAAGTCAGTG TTTGAGTGGG CACATGACAG GGGCCTTGTC     660

TGCCAGACGA ATGTAACTTG CTTCTTGAAG AACGAGAAAA ATCCTTACGA AGTGAATCAG     720

TCATTGAGGG AGAAGCAGTT GTATACGAAG AGTGATTCCT TGGACAACAT TGATTTTGGA     780

ACTACTCCAG ATGGGCCTAG CGATGCGTAC ATTGGACACT TAGACTACGG TGGTGGTGTG     840

GAGTGTGATG CACTATTCCC AGACTGGGGT CGACCAGACG TGGCTCAATG GTGGGGCGAT     900

AACTACAAGA AACTATTCAG CATTGGTCTC GACTTCGTAT GGCAAGACAT GAC           953
```

(2) INFORMATION FOR SEQ ID NO: 13:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 969 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
            (A) NAME/KEY: misc_difference
            (B) LOCATION: replace(814..821, "")
            (D) OTHER INFORMATION: /note= "Each g between (and
                    including) 814 and 821 is a misc. nucleic acid."

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
ATGACAAACT ACAACTACGA CAACTATAAC TACAACCAGT CAGATCTTAT TGCTCCAGGA      60
```

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```
TATCCTTCCG ACCCGAACTT CTACATTCCC ATGTATTTTG CAGCACCTTG GGTAGTTGTT    120

AAGGGATGCA GTGGCAACAG CGATGAACAG TACTCGTACG GATGGTTTAT GGATAATGTC    180

TCCCAAACTT ACATGAATAC TGGTGGTACT TCCTGGAACT GTGGAGAGGA GAACTTGGCA    240

TACATGGGAG CACAGTGCGG TCCATTTGAC CAACATTTTG TGTATGGTGA TGGAGATGGT    300

CTTGAGGATG TTGTCCAAGC GTTCTCTCTT CTGCAAGGCA AAGAGTTTGA GAACCAAGTT    360

CTGAACAAAC GTGCCGTAAT GCCTCCGAAA TATGTGTTTG GTTACTTTCA GGGAGTCTTT    420

GGGATTGCTT CCTTGTTGAG AGAGCAAAGA CCAGAGGGTG GTAATAACAT CTCTGTTTCA    480

GAGATTGTCG AAGGTTACCA AAGCAATAAC TTCCCTTTAG AGGGGTTAGC CGTAGATGTG    540

GATATGCAAC AAGATTTGCG GTGTAGTTCA CCACTGAAGA TTGAATTTTG GACGGCAAAT    600

AAGGTAGGCA CCGGGGGAGA CTCGAATAAC AAGTCGGTGT TTGAATGGGC ACATGACAAA    660

GGCCTTGTAT GTCAGACGAA TGTTACTTGC TTCTTGAGAA ACGACAACGG CGGGGCAGAT    720

TACGAAGTCA ATCAGACATT GAGGGAGAAG GGTTTGTACA CGAAGAATGA CTCACTGACG    780

AACACTAACT TCGGAACTAC CAACGACGGG CCGGGGGGGG GGTACATTGG ACATCTGGAC    840

TATGGTGGCG GAGGGAATTG TGATGCACTT TTCCCAGATT GGGGTCGACC GGGTGTGGCT    900

GAATGGTGGG GTGATAACTA CAGCAAGCTC TTCAAAATTG GTCTGGACTT CGTGTGGCAA    960

GATATGACA                                                            969
```

(2) INFORMATION FOR SEQ ID NO: 14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 607 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(128, "")
        (D) OTHER INFORMATION: /note= "g is a misc. nucleic acid"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(232, "")
        (D) OTHER INFORMATION: /note= "g is a misc. nucleic acid"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(249, "")

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(D) OTHER INFORMATION: /note= "g is a misc. nucleic acid"

(ix) FEATURE:
    (A) NAME/KEY: misc_difference
    (B) LOCATION: replace(526, "")
    (D) OTHER INFORMATION: /note= "g is a misc. nucleic acid"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATGACAAACT ACAATTACGA CAACTTGAAC TACAACCAAC CAGACGTCGT TCCTCCAGGT   60

TATCACGACC ATCCCAACTA CTACATTCCA ATGTACTACG CAGCACCGTG GTTGGTCGTT   120

CAGGGATGCG CGGGGACATC GAAGCAATAC TCGTACGGTT GGTTTATGGA CAATGTCTCT   180

CAGTCGTACA TGAACACTGG AGATACGGCG TGGAACTGCG GACAGGAAAA CGTGGCATAC   240

ATGGGCGCGC AATACGGGCC ATTTGATCAG CACTTTGTGT ATGGTGATGG AGATGGCCTT   300

GAAGATGTCG TCAAAGCGTT CTCCTTTCTT CAAGGAAAGG AGTTCGAAGA CAAAAAACTC   360

AACAAGCGTT CTGTAATGCC TCCGAAGTAC GTGTTTGGTT TCTTCCAGGG TGTTTTCGGT   420

GCACTTTCAC TGTTGAAGCA GAATCTGCCT GCCGGAGAGA ACAACATCTC AGTGCAAGAG   480

ATTGTGGAGG GTTACCAGGA TAACGACTAC CCCTTTGAAG GGCTCGCGGT AGATGTTGAT   540

ATGCAAGATG ATCTGCGAGT GTTTACTACC AAACCAGAAT ATTGGTCGGC AAACATGGTA   600

GGCGAAG   607

(2) INFORMATION FOR SEQ ID NO: 15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 90 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

    Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala
    1   5   10   15

    Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asn Asn Asp Ser
                20   25   30

    Asn Val Arg Arg Ala Gln Asn Asp His Phe Leu Leu Gly Gly His Asp
            35   40   45

    Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Ser Thr Glu
        50   55   60

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Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln Trp Tyr Lys Phe Gly Pro
65   70   75   80

Asp Phe Asp Thr Lys Pro Leu Glu Gly Ala
                85   90

(2) INFORMATION FOR SEQ ID NO: 16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N is T OR C"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(18, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(21, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

ATGTANAANA ANGANTCNAA NGT   23

(2) INFORMATION FOR SEQ ID NO: 17:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs

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          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)


  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(6, "")
          (D) OTHER INFORMATION: /note= "N IS T OR C"

  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(9, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"

  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(12, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"

  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(15, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"

  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(18, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"

  (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(21, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"


  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

ATGTANAAANA ANGANAGNAA NGT                                       23

(2) INFORMATION FOR SEQ ID NO: 18:

      (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 17 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: DNA (genomic)


     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(3, "")
```

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```
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

   (ix) FEATURE:                      .
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"


   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TANCCNTCNT GNCCNCC                                              17

(2) INFORMATION FOR SEQ ID NO: 19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: DNA (genomic)


   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(3, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

   (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
```

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    (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
    (A) NAME/KEY: misc_difference
    (B) LOCATION: replace(18, "")
    (D) OTHER INFORMATION: /note= "N IS C OR T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

GGNCCNAANT TNTACCANTG   20

(2) INFORMATION FOR SEQ ID NO: 20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(3, "")
        (D) OTHER INFORMATION: /note= "N IS T OR C"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

TANCGNTGGC ANGANGT   17

(2) INFORMATION FOR SEQ ID NO: 21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(3, "")
     (D) OTHER INFORMATION: /note= "N IS T OR C"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(6, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(12, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(15, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

TANAGNTGGC ANGANGT   17

(2) INFORMATION FOR SEQ ID NO: 22:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 71 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: double
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA

     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT TCTTGGCGGC   60

CACGACGGTT A   71

(2) INFORMATION FOR SEQ ID NO: 23:

     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 23 amino acids
         (B) TYPE: amino acid
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

```
Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe
1               5               10               15

Leu Leu Gly Gly His Asp Gly
            20
```

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 160 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

```
ATGTACAACA ACGACTCGAA CGTTCGCAGG GCGCAGAACG ATCATTTCCT TCTTGGTGGA      60

CATGATGGAT ATCGCATTCT GTGCGCGCCT GTTGTGTGGG AGAATTCGAC CGAACGGAAT      120

TGTACTTGCC CGTGCTGACC CAATGGTACA AATTCGGCCC                            160
```

(2) INFORMATION FOR SEQ ID NO: 25:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 54 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Met Tyr Asn Asn Asp Ser Asn Val Arg Arg Ala Gln Asn Asp His Phe
1               5               10               15

Leu Leu Gly Gly His Asp Gly Tyr Arg Ile Leu Cys Ala Pro Val Val
            20              25              30

Trp Glu Asn Ser Thr Glu Arg Glu Leu Tyr Leu Pro Val Leu Thr Gln
        35              40              45

Trp Tyr Lys Phe Gly Pro
        50
```

(2) INFORMATION FOR SEQ ID NO: 26:

    (i) SEQUENCE CHARACTERISTICS:

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     (A) LENGTH: 238 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: double
     (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
TACAGGTGGC AGGAGGTGTT GTACACTGCT ATGTACCAGA ATGCGGCTTT CGGGAAACCG      60

ATTATCAAGG CAGCTTCCAT GTACGACAAC GACAGAAACG TTCGCGGCGC ACAGGATGAC     120

CACTTCCTTC TCGGCGGACA CGATGGATAT CGTATTTTGT GTGCACCTGT TGTGTGGGAG     180

AATACAACCA GTCGCGATCT GTACTTGCCT GTGCTGACCA GTGGTACAAA TTCGGCCC      238
```

(2) INFORMATION FOR SEQ ID NO: 27:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 79 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: peptide

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

```
Tyr Arg Trp Gln Glu Val Leu Tyr Thr Ala Met Tyr Gln Asn Ala Ala
1               5                   10                  15

Phe Gly Lys Pro Ile Ile Lys Ala Ala Ser Met Tyr Asp Asn Asp Arg
            20              25                  30

Asn Val Arg Gly Ala Gln Asp Asp His Phe Leu Leu Gly Gly His Asp
            35              40                  45

Gly Tyr Arg Ile Leu Cys Ala Pro Val Val Trp Glu Asn Thr Thr Ser
    50              55                  60

Arg Asp Leu Tyr Leu Pro Val Leu Thr Lys Trp Tyr Lys Phe Gly
65              70              75
```

(2) INFORMATION FOR SEQ ID NO: 28:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 28 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: cDNA

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GCTCTAGAGC ATGTTTTCAA CCCTTGCG   28

(2) INFORMATION FOR SEQ ID NO: 29:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 36 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

AGCTTGTTAA CATGTATCCA ACCCTCACCT TCGTGG   36

(2) INFORMATION FOR SEQ ID NO: 30:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 34 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

ACAATTGTAC ATAGGTTGGG AGTGGAAGCA CCGC   34

(2) INFORMATION FOR SEQ ID NO: 31:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 75 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Lys Asn Leu His Pro Gln His Lys Met Leu Lys Asp Thr Val Leu Asp
1   5   10   15

Ile Val Lys Pro Gly His Gly Glu Tyr Val Gly Trp Gly Glu Met Gly
        20   25   30

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```
Gly Ile Gln Phe Met Lys Glu Pro Thr Phe Met Asn Tyr Phe Asn Phe
        35              40                  45

Asp Asn Met Gln Tyr Gln Gln Val Tyr Ala Gln Gly Ala Leu Asp Ser
    50              55              60

Arg Glu Pro Leu Tyr His Ser Asp Pro Phe Tyr
65              70              75
```

(2) INFORMATION FOR SEQ ID NO: 32:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(3, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OT T OR C"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(18, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(21, "")
        (D) OTHER INFORMATION: /note= "N IS C OR T"

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

CANCANAANA TGCTNAANGA NAC   23

(2) INFORMATION FOR SEQ ID NO: 33:

102 u

```
(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 23 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA


(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(3, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(6, "")
     (D) OTHER INFORMATION: /note= "N IS C OR T"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(9, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(15, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(18, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(21, "")
     (D) OTHER INFORMATION: /note= "N IS C OR T"


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:
```

CANCANAANA TGTTNAANGA NAC   23

```
(2) INFORMATION FOR SEQ ID NO: 34:

     (i) SEQUENCE CHARACTERISTICS:
          (A) LENGTH: 20 base pairs
          (B) TYPE: nucleic acid
          (C) STRANDEDNESS: single
          (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA


     (ix) FEATURE:
```

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```
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(3, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

 (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

 (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

 (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

 (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

 (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(18, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"


 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TANAANGGNT CNCTNTGNTA                                        20

(2) INFORMATION FOR SEQ ID NO: 35:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(3, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

    (ix) FEATURE:
```

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```
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(9, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(12, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

(ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(15, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"

(ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(18, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A"


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:
```

TANAANGGNT CNGANTGNTA   20

(2) INFORMATION FOR SEQ ID NO: 36:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 37 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA



    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
```

AAACTGCAGC TGGCGCGCCA TGGCAGGATT TTCTGAT   37

(2) INFORMATION FOR SEQ ID NO: 37:

```
    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA


    (ix) FEATURE:
        (A) NAME/KEY: misc_difference
        (B) LOCATION: replace(6, "")
        (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"
```

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(ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(9, "")
      (D) OTHER INFORMATION: /note= "N IS C OR T"

(ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(12, "")
      (D) OTHER INFORMATION: /note= "N IS C OR T"

(ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(15, "")
      (D) OTHER INFORMATION: /note= "N IS C OR T"

(ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(18, "")
      (D) OTHER INFORMATION: /note= "N IS C OR T"

(ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(21, "")
      (D) OTHER INFORMATION: /note= "N IS C OR T"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

ATGACNAANT ANAANTANGA NAA   23

(2) INFORMATION FOR SEQ ID NO: 38:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 21 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(1, "")
      (D) OTHER INFORMATION: /note= "N IS A OR G"

    (ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(4, "")
      (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

    (ix) FEATURE:
      (A) NAME/KEY: misc_difference
      (B) LOCATION: replace(13, "")
      (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

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```
(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(16, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

(ix) FEATURE:
     (A) NAME/KEY: misc_difference
     (B) LOCATION: replace(19, "")
     (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"


(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
```

NTGNGGCATC ATNGCNGGNA C   21

(2) INFORMATION FOR SEQ ID NO: 39:

```
     (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 23 base pairs
         (B) TYPE: nucleic acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: cDNA


     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(6, "")
          (D) OTHER INFORMATION: /note= "N IS G OR A"

     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(9, "")
          (D) OTHER INFORMATION: /note= "N IS C OR T"

     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(15, "")
          (D) OTHER INFORMATION: /note= "N IS G OR A OR T OR C"

     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(18, "")
          (D) OTHER INFORMATION: /note= "N IS G OR A"

     (ix) FEATURE:
          (A) NAME/KEY: misc_difference
          (B) LOCATION: replace(21, "")
          (D) OTHER INFORMATION: /note= "N IS G OR A"


     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:
```

GTCATNTCNT GCCANACNAA NTC   23

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description
on page ____14____ , line ____16____

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☐
Name of depositary institution
The National Collections of Industrial and Marine Bacteria Limited (NCIMB)
Address of depositary institution *(including postal code and country)*   23 St. Machar Drive   Aberdeen   Scotland   AB2 1RY   United Kingdom

Date of deposit 20 JUNE 1994	Accession Number NCIMB 40652

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*   This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R.M. HENDRIKMAKER	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description
on page ___14___ , line ___18___

B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit 20 JUNE 1994	Accession Number NCIMB 40653

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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Authorized officer R.M. WANDEMAKER	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description
on page ___14___ , line ___25___

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☐

Name of depositary institution

Culture Collection of Algae and Protozoa (CCAP)

Address of depositary institution (*including postal code and country*)

Dunstaffnage Marine Laboratory
P.O. Box 3
Oban
Argyll   PA34 4AD
Scotland   United Kingdom

Date of deposit 11 OCTOBER 1994	Accession Number CCAP 1373/1

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*)   This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has b en refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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Authorized officer R.M. MANDEMAKER	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page _____ 15 _____ , line _____ 6 _____

B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit 3 OCTOBER 1994	Accession Number NCIMB 40687

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*	This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*
The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description

on page ___ 15 ___ , line ___ 8 ___

B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
3 OCTOBER 1994	NCIMB 40688

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

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Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page **15**, line **10**	

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (including postal code and country)

23 St. Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
3 OCTOBER 1994	NCIMB 40689

C. ADDITIONAL INDICATIONS (leave blank if not applicable)   This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description
on page ___**16**___ , line ___**13**___

B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet ☐

Name of depositary institution

Culture Collection of Algae and Protozoa (CCAP)

Address of depositary institution *(including postal code and country)*

Dunstaffnage Marine Laboratory
P.O. Box 3
Oban
Argyll   PA34 4AD
Scotland   United Kingdom

Date of deposit	Accession Number
11 OCTOBER 1994	CCAP 1373/2

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)*	This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any
other designated state having equivalent legislation, a sample of the deposited
microorganism will be made available until the publication of the mention of the
grant of the European patent or until the date on which the application has been
refused or withdrawn or is deemed to be withdrawn, only by the issue of such a
sample to an expert nominated by the person requesting the sample. (Rule 28(4)
EPC).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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CLAIMS

1. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α-1,4-glucan with the enzyme α-1,4-glucan lyase characterised in that enzyme is used
5   in substantially pure form.

2. A method according to claim 1 wherein if the glucan contains links other than and in addition to the α-1,4- links the α-1,4-glucan lyase is used in conjunction with a suitable reagent that can break the other links.
10

3. A method according to claim 2 wherein the glucan is starch and a hydrolase, preferably a glucanohydrolase, is used in conjunction with the α-1,4-glucan lyase.

4. A method according to claim 2 or claim 3 wherein the hydrolase is at least one of
15   pullanase or isoamylase.

5. A method according to any preceding claim wherein the α-1,4-glucan lyase is bound to a support or, more preferably, is in a dissolved form.

20   6. A method according to any preceding claim wherein the enzyme is isolated from either a fungus, preferably *Morchella costata* or *Morchella vulgaris*, or from a fungally infected algae, preferably *Gracilariopsis lemaneiformis* or from algae alone, preferably *Gracilariopsis lemaneiformis*.

25   7. A method according to claim 6 wherein the enzyme is isolated and/or further purified from the fungus or from the fungally infected algae or from algae alone using a gel that is not degraded by the enzyme.

8. A method according to claim 7 wherein the gel is based on dextrin or derivatives
30   thereof, preferably the gel is a cyclodextrin - more preferably beta-cyclodextrin.

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9. A method according to any of the preceding claims wherein the enzyme comprises the amino acid sequence SEQ. ID. No. 1. or the amino acid sequence SEQ. ID. No. 2 or the amino acid sequence SEQ. ID. No. 5. or the amino acid sequence SEQ. ID. No. 6, or any variant thereof.

10. A method according to any preceding claim wherein the enzyme is obtained from the expression of a nucleotide sequence coding for the enzyme.

11. A method according to claim 10 wherein the nucleotide sequence is a DNA sequence.

12. A method according to claim 11 wherein the DNA sequence comprises a sequence that is the same as, or is complementary to, or has substantial homology with, or contains any suitable codon substitutions for any of those of, SEQ. ID. No. 3 or SEQ. ID. No. 4 or SEQ. ID. No. 7 or SEQ. ID. No. 8.

13. The method according to claim 3 or any claim dependent thereon wherein the starch is used in high concentration - such as up to about 25% solution.

14. The method according to any one of the preceding claims wherein the substrate is treated with the enzyme in the presence of a buffer.

15. The method according to any one of claims 1 to 13 wherein the substrate is treated with the enzyme in the presence of at least substantially pure water.

16. The method according to any one of the preceding claims wherein the substrate is treated with the enzyme in the absence of a co-factor.

17. The method according to any one of the preceding claims wherein the enzyme is used in combination with amylopectin or dextrin.

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18. A method of preparing the sugar 1,5-D-anhydrofructose comprising treating an α-1,4-glucan with the enzyme α-1,4-glucan lyase characterised in that enzyme comprises the amino acid sequence SEQ. ID. No. 1. or the amino acid sequence SEQ. ID. No. 2 or the amino acid sequence SEQ. ID. No. 5. or the amino acid

5   sequence SEQ. ID. No. 6, or any variant thereof.

19. The sugar 1,5-D-anhydrofructose when prepared by the method of the present invention.

10   20. The use of a reagent that can increase the hydrophobicity of the reaction medium to increase the stability and activity of the GL enzyme.

21. Use of AF as an anti-oxidant.

15   22. Use of AF as a sweetener.

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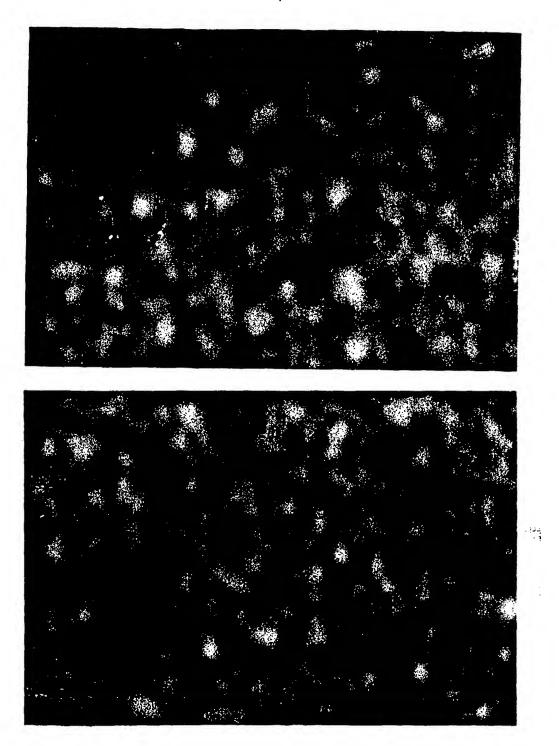
Fig.1. Calcoflour White stainings revealing fungi in upper part and lower part of Graci-laria lemnaeformis. (108x and 294x).
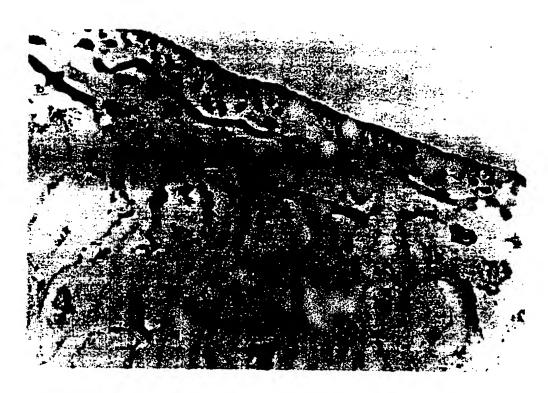
Fig.2. PAS / Anilinblue Black staining of Gracilaria lemnaeformis with fungi. The fungi have a significant higher content of carbohydrates.

Fig. 3. The micrograph shows longitudinal and grazing sections of two thin-walled fungal
hypha (f) growing between thick walls (w) of algal cells. Note thylacoid membranes in
the algal chloroplast (arrows)
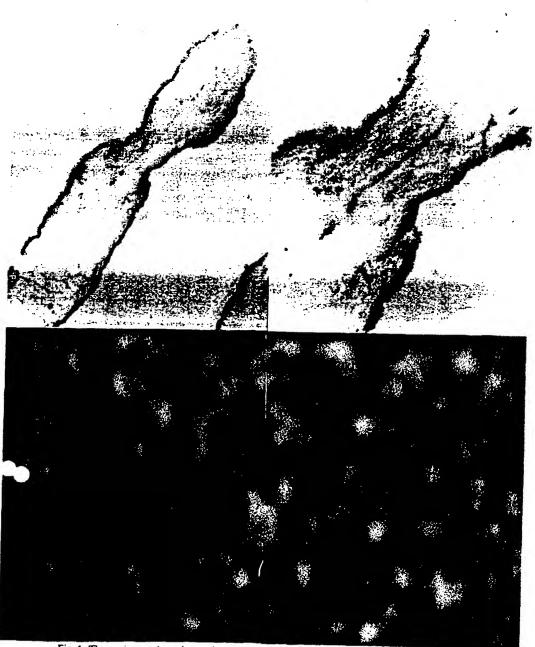
Fig.4. The antisense detections with clone 2 probe (upper row) are restricted to the fungi illustrated by the Calcofluor White staining of the succeeding section (lower row). (46x and 108x).

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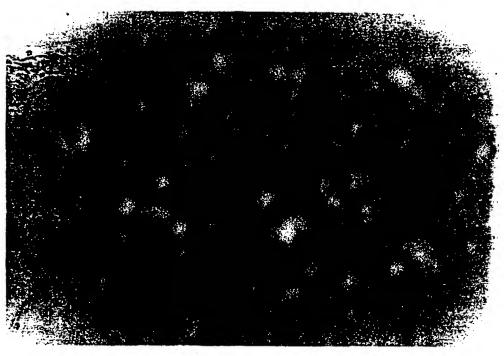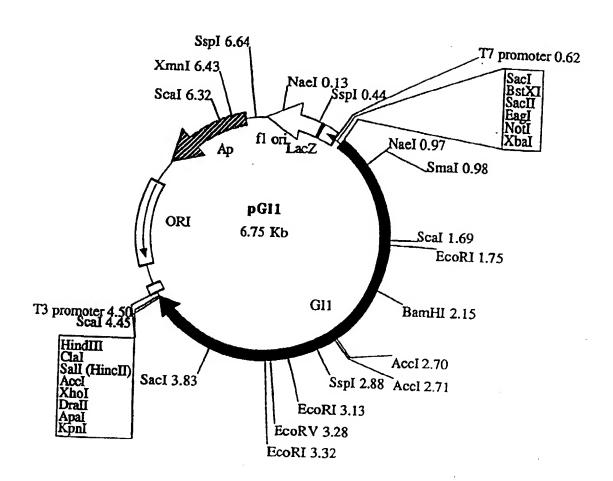
Fig.5. Intense antisense detections with clone 2 probe are found over the fungi in Gracilaria lemnaeformis (294x).

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FIG. 6.

Fig. 7.

T7 promoter 0.65

KpnI
ApaI
DraII
XhoI
AccI
SalI(HincII)
ClaI
HindIII

SspI 6.42
XmnI 6.21
ScaI 6.10
SspI 0.02
NaeI 0.33
HpaI 0.72
f1 ori
LacZ
Ap
XhoI 1.10
AccI 1.14
BamHI 1.23
NaeI 1.40
SalI 1.44
ORI
pGl2
6.53 Kb
EcoRV 1.66
ScaI 1.75
LacI
Gl2
T3 promoter 4.34
SacI 4.33
AccI 2.48
XhoI 3.89
SmaI 3.03
EcoRI 3.18
EcoRV 3.34
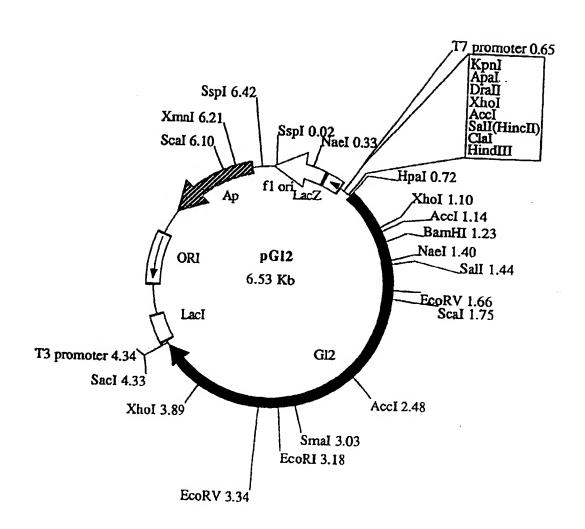
FIGURE 8

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MFSTLAFVAP  SALGASTFVG  AEVRSNVRIH  SAFPAVHTAT  RKTNRLNVSM  TALSDKQTAT  AGSTDNPDGI
DYKTYDYVGV  WGFSPLSNTN  WFAAGSSTPG  GITDWTATMN  VNFDRIDNPS  ITVQHPVQVQ  VTSYNNNSYR
VRFNPDGPIR  DVTRGPILKQ  QLDWIRTQEL  SEGCDPGMTF  TSEGFLTFET  KDLSVIIYGN  FKTRVTRKSD
GKVIMENDEV  GTASSGNKCR  GLMFVDRLYG  NAIASVNKNF  RNDAVKQEGF  YGAGEVNCKY  QDTYILERTG
IAMTNYNYDN  LNYNQWDLRP  PHHDGALNPD  YYIPMYYAAP  WLIVNGCAGT  SEQYSYGWFM  DNVSQSYMNT
GDTTWNSGQE  DLAYMGAQYG  PFDQHFVYGA  GGGMECVVTA  FSLLQGKEFE  NQVLNKRSVM  PPKYVFGFFQ
GVFGTSSLLR  AHMPAGENNI  SVEEIVEGYQ  NNNFPFEGLA  VDVDMQDNLR  VFTTKGEFWT  ANRVGTGGDP
NNRSVFEWAH  DKGLVCQTNI  TCFLRNDNEG  QDYEVNQTLR  ERQLYTKNDS  LTGTDFGMTD  DGPSDAYIGH
LDYGGGVECD  ALFPDWGRPD  VAEWWGNNYK  KLFSIGLDFV  WQDMTVPAMM  PHKIGDDINV  KPDGNWPNAD
DPSNGQYNWK  TYHPQVLVTD  MRYENHGREP  MVTQRNIHAY  TLCESTRKEG  IVENADTLTK  FRRSYIISRG
GYIGNQHFGG  MWVGDNSTTS  NYIQMMIANN  INMNMSCLPL  VGSDIGGFTS  YDNENQRTPC  TGDLMVRYVQ
AGCLLPWFRN  HYDRWIESKD  HGKDYQELYM  YPNEMDTLRK  FVEFRYRWQE  VLYTAMYQNA  AFGKPIIKAA
SMYNNDSNVR  RAQNDHFLLG  GHDGYRILCA  PVVWENSTER  ELYLPVLTQW  YKFGPDFDTK  PLEGAMNGGD
RIYNYPVPQS  ESPIFVREGA  ILPTRYTLNG  ENKSLNTYTD  EDPLVFEVFP  LGNNRADGMC  YLDDGGVTTN
AEDNGKFSVV  KVAAEQDGGT  ETITFTNDCY  EYVFGGPFYV  RVRGAQSPSN  IHVSSGAGSQ  DMKVSSATSR
AALFNDGENG  DFWVDQETDS  LWLKLPNVVL  PDAVITIT
```

FIGURE 9

```
GL1   - MFSTLAFVAPSALGASTFVGAEV-RSNVRIHSAFPAVHTATRKTNRLNVS  -49
          :. ::.:::::::::: ::     . :: . ::: :::  : ::.:::::
GL2   - MYPTLTFVAPSALGARTFTCVGIFRSHILIHSVVPAVRLAVRKSNRLNVS  -50

GL1   - MTALSDKQTATAGSTDNPDGIDYKTYDYVGVWGFSPLSNTNWFAAGSSTP  -99
          :.:: :: :: .: :::: :.: ::::: :: : :::::::::::::
GL2   - MSALFDKPTAVTGGKDNPDNINYTTYDYVPVWRFDPLSNTNWFAAGSSTP  -100

GL1   - GGITDWTATMNVNFDRIDNPSITVQHPVQVQVTSYNNNSYRVRFNPDGPI  -149
          : : :::::::::::::::::: :.. ::::::::: :: .:::::::::
GL2   - GDIDDWTATMNVNFDRIDNPSFTLEKPVQVQVTSYKNNCFRVRFNPDGPI  -150

GL1   - RDVTRGPILKQQLDWIRTQELSEGCDPGMTFTSEGFLTFETKDLSVIIYG  -199
          ::: ::::: ::: ::: :: : :: : :: :::: :::::: :::::
GL2   - RDVDRGPILQQQLNWIRKQEQSKGFDPKMGFTKEGFLKFETKDLNVIIYG  -200

GL1   - NFKTRVTRKSDGKVIMENDEVGTASSGNKCRGLMFVDRLYGNAIASVNKN  -249
          :::::::::: ::: :::: :: . : ::::::::::::::  :::::: :
GL2   - NFKTRVTRKRDGKGIMENNEVPAGSLGNKCRGLMFVDRLYGTAIASVNEN  -250

GL1   - FRNDAVKQEGFYGAGEVNCKYQDT------YILERTGIAMTNYNYDNLNY  -293
          .::: . :::::::::: . :.     :::::::::::::::: ::
GL2   - YRNDPDRKEGFYGAGEVNCEFWDSEQNRNKYILERTGIAMTNYNYDNYNY  -300

GL1   - NQWDLRPPHHDGALNPDYYIPMYYAAPWLIVNGCAGTS-EQYSYGWFMDN  -342
          :: :: :   : .:::::.::::..: ::.: :::::: : :::::::::
GL2   - NQSDLIAP--GYPSDPNFYIPMYFAAPWVVVKGCSGNSDEQYSYGWFMDN  -348

GL1   - VSQSYMNTGDTTWNSGQEDLAYMGAQYGPFDQHFVYGAGGGMECVVTAFS  -392
          :::.:::::: :.:: : :::::::: :::::::::::: ::::: :::::
GL2   - VSQTYMNTGGTSWNCGEENLAYMGAQCGPFDQHFVYGDGDGLEDVVQAFS  -398

GL1   - LLQGKEFENQVLNKRSVMPPKYVFGFFQGVFGTSSLLRAHMPAGENNISV  -442
          :::::::::::::::::.::::::::::.:::::: : : :::::
GL2   - LLQGKEFENQVLNKRAVMPPKYVFGYFQGVFGIASLLREQRPEGGNNISV  -448

GL1   - EEIVEGYQNNNFPFEGLAVDVDMQDNLRVFTTKGEFWTANRVGTGGDPNN  -492
          .:::::: :::: :::::::::::::: :::::::: :::::::.:::: ::
GL2   - QEIVEGYQSNNFPLEGLAVDVDMQQDLRVFTTKIEFWTANKVGTGGDSNN  -498

GL1   - RSVFEWAHDKGLVCQTNITCFLRNDNEGQDYEVNQTLRERQLYTKNDSLT  -542
          :::::::::::::::::::.:::::::: : :::::::::: :::::::::
GL2   - KSVFEWAHDKGLVCQTNVTCFLRNDNGGADYEVNQTLREKGLYTKNDSLT  -548

GL1   - GTDFGMTDDGPSDAYIGHLDYGGGVECDALFPDWGRPDVAEWWGNNYKKL  -592
          : :: :::::::::::::::::::: :::::::::: ::::::: :: ::
GL2   - NTNFGTTNDGPSDAYIGHLDYGGGGNCDALFPDWGRPGVAEWWGDNYSKL  -598

GL1   - FSIGLDFVWQDMTVPAMMPHKIGDDINVKPDGNWPNADDPSNGQYNWKTY  -642
          : ::::::::::::::::::::.::  .    ::: :::::: :::::.:
GL2   - FKIGLDFVWQDMTVPAMMPHKVGDAVDTRSPYGWPNENDPSNGRYNWKSY  -648

GL1   - HPQVLVTDMRYENHGREPMVTQRNIHAYTLCESTRKEGIVENADTLTKFR  -692
          :::::::::::::::::::::::::: ::::::::::::::::::::::::
GL2   - HPQVLVTDMRYENHGREPMFTQRNMHAYTLCESTRKEGIVANADTLTKFR  -698
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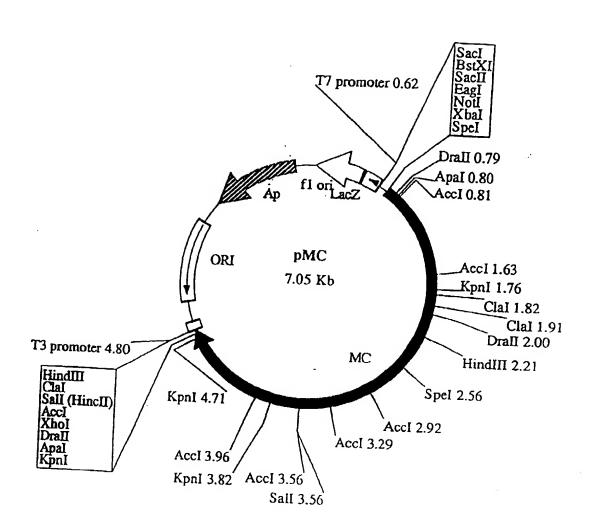
FIGURE 9 continued

```
GL1    - RSYIISRGGYIGNQHFGGMWVGDNSTTSNYIQMMIANNINMNMSCLPLVG -742
         :::::::::::::::::::::::::::..  :.::::::: ,:::::::::::
GL2    - RSYIISRGGYIGNQHFGGMWVGDNSSSQRYLQMMIANIVNMNMSCLPLVG -748

GL1    - SDIGGFTSYDNENQRTPCTGDLMVRYVQAGCLLPWFRNHYDRWIESKDHG -792
         :::::::::::    :  : :::::::.:::::::::::: : .: :  :
GL2    - SDIGGFTSYDG---RNVCPGDLMVRFVQAGCLLPWFRNHYGRLVEGKQEG -795

GL1    - KDYQELYMYPNEMDTLRKFVEFRYRWQEVLYTAMYQNAAFGKPIIKAASM -842
         : :::::::  :: :::::.:::::::::::::::::::::::::::::::
GL2    - KYYQELYMYKDEMATLRKFIEFRYRWQEVLYTAMYQNAAFGKPIIKAASM -845

GL1    - YNNDSNVRRAQNDHFLLGGHDGYRILCAPVVWENSTERELYLPVLTQWYK -892
         : :: ::: :: :::::::::::::::::::::.: :.:::::: :::
GL2    - YDNDRNVRGAQDDHFLLGGHDGYRILCAPVVWENTTSRDLYLPVLTKWYK -895

GL1    - FGPDFDTKPLEGAMNGGDRIYNYPVPQSESPIFVREGAILPTRYTLNGEN -942
         :::::.::: :. :. ::   : :: ::::.::::::::::::: : :
GL2    - FGPDYDTKRLDSALDGGQMIKNYSVPQSDSPIFVREGAILPTRYTLDGSN -945

GL1    - KSLNTYTDEDPLVFEVFPLGNNRADGMCYLDDGGVTTNAEDNGKFSVVKV -992
         ::.:::: :::::::::::::::::::::: ::: :::::. :
GL2    - KSMNTYTDKDPLVFEVFPLGNNRADGMCYLDDGGITTDAEDHGKFSVINV -995

GL1    - AAEQDGGTETITFTNDCYEYVFGGPFYVRVRGAQSPSNIHVSSGAGSQDM -1042
         :   : :: :: :.: : ::: ::::::. : :: ::::::: ::
GL2    - EALRKGVTTTIKFAYDTYQYVFDGPFYVRIRNLTTASKINVSSGAGEEDM -1045

GL1    - KVSSATSRAALFNDGENGDFWVDQETDSLWLKLPNVVLPDAVITIT -1088
         .:: :::::: :: :..: ::.: :::.::::.:::::::::
GL2    - TPTSANSRAALFSDGGVGEYWADNDTSSLWMKLPNLVLQDAVITIT -1091
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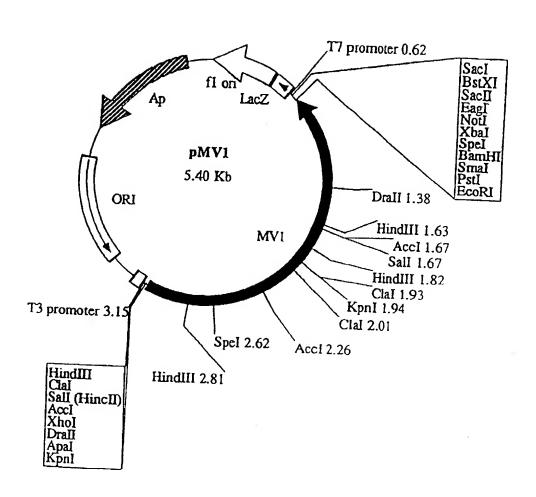
Figure 10. Microphotograph of a fungal hypha (f) growing bet-
ween algal cell walls (w). Note grains of floridean starch (s)
and thylakoids (arrows) in the algal cell. Bar = 2 μm.

Fig 11

Fig 12

Fig13

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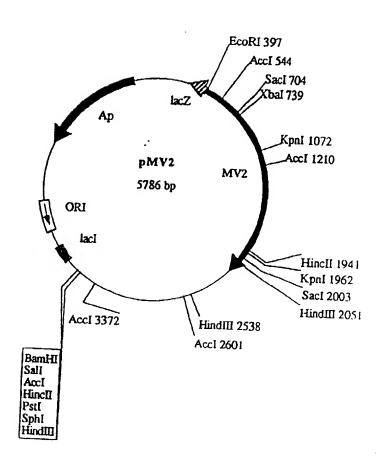
FIGURE 14

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              10        20        30        40        50        60
               |         ·|         |         |         |      · |
     1 AGACAGGTGC GTTTTTGTTT ATTCTATTCT GTGCGGCAGA TATGCACTCA CAAGAAACAA
    61 ATTGTACAAA TATTTCTAAT TACAGTTGTA GGTGCAGTTG AAAATCCGGT CGCACAAAGA
   121 TCATTGATGC ACAAAGATGA TAACGCCTGA TTAGTACTCA AGGTTTAATT GGGTATGTGT
   181 GCGACCTCTC TTTGGCTAGC ATTACCTGAT TGGTTACAAC TGCAAATACT GCGGCAGCAA
   241 TGAGGAATGA AGTCAGCATC GATAGCTCGG CCTCATAAAA ATTGATTTCA ATTTTATATT
   301 CCCAGTTTTA ATCTCGAATC CTATATAATG GCCATCGTTC CCTCCTCGCC TCTTCATTCT
   361 CCTCCATCAC TCCAGCTCAG TCATCCCTCA ACTTGGCCTC CTCTGATATC TTCCGAACAA
   421 AACATCTTGT CCAATCTTTT TTTGAGCTAG ATCTCATTAT ACCTCCGTCA TGGCAGGATT
   481 TTCTGATCCT CTCAACTTTT GCAAAGCAGA AGACTACTAC AGTGTTGCGC TAGACTGGAA
   541 GGGCCCTCAA AAAATCATTG GAGTAGACAC TACTCCTCCA AAGAGCACCA AGTTCCCCAA
   601 AAACTGGCAT GGAGTGAACT TGAGATTCGA TGATGGGACT TTAGGTGTGG TTCAGTTCAT
   661 TAGGCCGTGC GTTTGGAGGG TTAGATACGA CCCTGGTTTC AAGACCTCTG ACGAGTATGG
   721 TGATGAGAAT ACGTGAGTTA CCCCATATGT CATTATTGGT AGCGAAAAAC ATATGCTAAT
   781 CAACTAACGA GGCATATAGG AGGACAATTG TGCAAGATTA TATGAGTACT CTGAGTAATA
   841 AATTGGATAC TTATAGAGGT CTTACGTGGG AAACCAAGTG TGAGGATTCG GGAGATTTCT
   901 TTACCTTCTC AGTAAGTGCC AGTACTGCTA TAGCTCCGCT ATATATATAA CACCACTAAC
   961 TAACTGCCCT AAATAGTCCA AGGTCACCGC CGTTGAAAAA TCCGAGCGGA CCCGCAACAA
  1021 GGTCGGCGAT GGCCTCAGAA TTCACCTATG GAAAAGCCCT TTCCGCATCC AAGTAGTGCG
  1081 CACCTTGACC CCTTTGAAGG ATCCTTACCC CATTCCAAAT GTAGCCGCAG CCGAAGCCCG
  1141 TGTGTCCGAC AAGGTCGTTT GGCAAACGTC TCCCAAGACA TTCAGAAAGA ACCTGCATCC
  1201 GCAACACAAG ATGCTAAAGG ATACAGTTCT TGACATTGTC AAACCTGGAC ATGGCGAGTA
  1261 TGTGGGGTGG GGAGAGATGG GAGGTATCCA GTTTATGAAG GAGCCAACAT TCATGAACTA
  1321 TTTTAGTAAG CCCCGAAGAG GTTCCTTATA AATTCTTGGT GGTCATTTTT ACTAACCCAG
  1381 TGTAGACTTC GACAATATGC AATACCAGCA AGTCTATGCC CAAGGTGCTC TCGATTCTCG
  1441 CGAGCCACTG TAAGTACCGT CCTGTGGCAC GACTTAACCC AATAACTAAT CTTTCAACAA
  1501 GGTACCACTC GGATCCCTTC TATCTTGATG TGAACTCCAA CCCGGAGCAC AAGAATATCA
```

FIGURE 14 CONTINUED

```
1561 CGGCAACCTT TATCGATAAC TACTCTCAAA TTGCCATCGA CTTTGGAAAG ACCAACTCAG
1621 GCTACATCAA GCTGGGAACC AGGTATGGTG GTATCGATTG TTACGGTATC AGTGCGGATA
1681 CGGTCCCGGA AATTGTACGA CTTTATACAG GTCTTGTTGG ACGTTCAAAG TTGAAGCCCA
1741 GATATATTCT CGGGGCCCAT CAAGCCTGTA AGTCCTTCCC CTCATGAGTG ATTTATCAGA
1801 CTTGCATAAT AAACTAACCT CGTTTTCAAA GGTTATGGAT ACCAACAGGA AAGTGACTTG
1861 TATTCTGTGG TCCAGCAGTA CCGTGACTGT AAATTTCCAC TTGACGGGAT TCACGTCGAT
1921 GTCGATGTTC AGGTAAATGG CCATGGTATC ATTGAAGCTT TGAGAAATGT TCTAACTGTG
1981 TTTATAACAT TCCTAGGACG GCTTCAGAAC TTTCACCACC AACCCACACA CTTTCCCTAA
2041 CCCCAAAGAG ATGTTTACTA ACTTGAGGAA TAATGGAATC AAGTGCTCCA CCAATATCAC
2101 TCCTGTTATC AGCATTAACA ACAGAGAGGG TGGATACAGT ACCCTCCTTG AGGGAGTTGA
2161 CAAAAAATAC TTTATCATGG ACGACAGATA TACCGAGGGA ACAAGTGGGA ATGCGAAGGA
2221 TGTTCGGTAC ATGTACTACG GTGGTGGTAA TAAGGTTGAG GTCGATCCTA ATGATGTTAA
2281 TGGTCGGCCA GACTTTAAAG ACAACTAGTA AGTTGTTTAT TTGACTACGA TAGGTAACCC
2341 GTAAGCGGCA TTAACATATT TGTAGTGACT TCCCCGCGAA CTTCAACAGC AAACAATACC
2401 CCTATCATGG TGGTGTGAGC TACGGTTATG GGAACGGTAG TGTAAGTGAC GATATCTCAC
2461 CAACATAATG AAATTTATAA GGACTAACTA GACACAAAAA TTTGTAGGCA GGTTTTTACC
2521 CGGACCTCAA CAGAAAGGAG GTTCGTATCT GGTGGGGAAT GCAGTACAAG TATCTCTTCG
2581 ATATGGGACT GGAATTTGTG TGGCAAGACA TGACTACCCC AGCAATCCAC ACATCATATG
2641 GAGACATGAA AGGGTTGCCC ACCCGTCTAC TCGTCACCTC AGACTCCGTC ACCAATGCCT
2701 CTGAGAAAAA GCTCGCAATT GAAACTTGGG CTCTCTACTC CTACAATCTC CACAAAGCAA
2761 CTTGGCATGG TCTTAGTCGT CTCGAATCTC GTAAGAACAA ACGAAACTTC ATCCTCGGGC
2821 GTGGAAGTTA TGCCGGAGCC TATCGTTTTG CTGGTCTCTG GACTGGGGAT AATGCAAGTA
2881 ACTGGGAATT CTGGAAGATA TCGGTCTCTC AAGTTCTTTC TCTGGGCCTC AATGGTGTGT
2941 GCATCGCGGG GTCTGATACG GGTGGTTTTG AACCCTACCG TGATGCAAAT GGGGTCGAGG
3001 AGAAATACTG TAGCCCAGAG CTACTCATCA GGTGGTATAC TGGTTCATTC CTCTTGCCGT
3061 GGCTCAGGAA CCATTATGTC AAAAAGGACA GGAAATGGTT CCAGGTAATC TATCCTTTCT
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FIGURE 14 CONTINUED

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3121 TATCTTTGAA GCATTGAAGA TACTAAGATA TAATCTAGGA ACCATACTCG TACCCCAAGC
3181 ATCTTGAAAC CCATCCAGAA CTCGCAGACC AAGCATGGCT CTATAAATCC GTTTTGGAGA
3241 TCTGTAGGTA CTATGTGGAG CTTAGATACT CCCTCATCCA ACTACTTTAC GACTGCATGT
3301 TTCAAAACGT AGTCGACGGT ATGCCAATCA CCAGATCTAT GGTATGTATT CTACCCTAGG
3361 CTTCCAGAGC AACATATGCT AACCAATTGA ACCTGGGTTT CTAGCTCTTG ACCGATACTG
3421 AGGATACCAC CTTCTTCAAC GAGAGCCAAA AGTTCCTCGA CAACCAATAT ATGGCTGGTG
3481 ACGACATTCT TGTTGCACCC ATCCTCCACA GTCGCAAAGA AATTCCAGGC GAAAACAGAG
3541 ATGTCTATCT CCCTCTTTAC CACACCTGGT ACCCCTCAAA TTTGAGACCA TGGGACGATC
3601 AAGGAGTCGC TTTGGGGAAT CCTGTCGAAG GTGGTAGTGT CATCAATTAT ACTGCTAGGA
3661 TTGTTGCACC CGAGGATTAT AATCTCTTCC ACAGCGTGGT ACCAGTCTAC GTTAGAGAGG
3721 GTAAGCAGTA AAATAATCTC TTCCCAGTTT CAAATACATT TAGCTAGTAG CTAACGCTAT
3781 GAACCTACAG GTGCCATCAT CCCGCAAATC GAAGTACGCC AATGGACTGG CCAGGGGGGA
3841 GCCAACCGCA TCAAGTTCAA CATCTACCCT GGAAAGGATA AGGTAAAATT CAATGATCAC
3901 CCTGCATCTA TTCCATCGCT GGTTTTCTTT ACCCTTACTG ACTTCATTCC TCAAAATACA
3961 GGAGTACTGT ACCTATCTTG ATGATGGTGT TAGCCGTGAT AGTGCGCCGG AAGACCTCCC
4021 ACAGTACAAA GAGACCCACG AACAGTCGAA GGTTGAAGGC GCGGAAATCG CAAAGCAGAT
4081 TGGAAGAAG ACGGGTTACA ACATCTCAGG AACCGACCCA GAAGCAAAGG GTTATCACCG
4141 CAAAGTTGCT GTCACACAAG TAATACCGCC CTTGACTTGT ATCACTTCCT GACATCATGC
4201 TAATATTTCT CTGTTTACCT CAAAGACGTC AAAAGACAAG ACGCGTACTG TCACTATTGA
4261 GCCAAAACAC AATGGATACG ACCCTTCCAA AGAGGTGGGT GATTATTATA CCATCATTCT
4321 TTGGTACGCA CCAGGTTTCG ATGGCAGCAT CGTCGATGTG AGCAAGACGA CTGTGAATGT
4381 TGAGGGTGGG GTGGAGCACC AAGTTTATAA GAACTCCGAT TTACATACGG TTGTTATCGA
4441 CGTGAAGGAG GTGATCGGTA CCACAAAGAG CGTCAAGATC ACATGTACTG CCGCTTAAGG
4501 TCTTTTCTTG GGGGCGGGAG GCGAGACCTT CGAAATGTAT ACGGGAGTGG TAACTCCGGG
4561 AAAATGGTGA TATGGGGGAT CAAGTTGGAG GGGAATCTGT TTATTTCTTT ATTTCTTTAT
4621 TTACTGGATT GGAAAATAGG GAGCACAGTT CTGACTGGAT TGGTTTGATT GTTGGCCTCT
4681 ACGGGTTCTC TTTACTTTGT CTGGAAATCC AATTTATTGT TATGCG
```

FIGURE 15   18/26

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              10        20        30        40        50        60
               |         |         |         |         |         |
   1 ATGCAGGCAA CGACAGGCGT TTTTTGTTTT ATCCGCAGAG GTGCAGCAGC AGGAAACAAA
  61 CCATACAAAC ATTCCTTGAC GCGGTTTTAG GTGCAGTTAA GGCCCGGGCG CACCAAGAAC
 121 ATTGATGTAC TTGGTCTAAA AAAGATCATA ATACCCGATT AGTGTTCATG GTTTGATTGG
 181 GTCTAAGTAC AAGTTTTACA GAGTTCAGCT TAGTTCATTG TTCGAAACTA CCAATATCAC
 241 ACCTATGCCT GCTGGCATTG ATAGCTCGGC TTGTGAAAGC TGATTACAAT CTTACATTTC
 301 TGATTTAATA TCGGACTGAT CTATATATAA GGGTCATCAT TTCCTCTCCG CCTTTTGGTT
 361 CTCTTTCATC ACCCCAGCCC AATCATCACC GTTGGCCTTT ACTTCTCTCT TCCGTTGATA
 421 TTTTCTCGAC AAAACATCTT GTCCACTGTT AGGCTAGCTC CCAGAATTAT CCCTCCAACA
 481 TGGCAGGATT ATCCGACCCT CTCAATTTCT GCAAAGCAGA GGACTACTAC GCTGCTGCCA
 541 AAGGCTGGAG TGGCCCTCAG AAGATCATTC GCTATGACCA GACCCCTCCT CAGGGTACAA
 601 AAGATCCGAA AAGCTGGCAT GCGGTAAACC TTCCTTTCGA TGACGGGACT ATGTGTGTAG
 661 TGCAATTCGT CAGACCCTGT GTTTGGAGGG TTAGATATGA CCCCAGTGTC AAGACTTCTG
 721 ATGAGTACGG CGATGAGAAT ACGTGGGTCG CCCAGTCAAT TAACTATGCC GCTAGTGATT
 781 ATGGAAAGCT TCTGCTAACC GATCAATGAG GCATGTAGGA GGACTATTGT ACAAGACTAC
 841 ATGACTACTC TGGTTGGAAA CTTGGACATT TTCAGAGGTC TTACGTGGGT TTCTACGTTG
 901 GAGGATTCGG GCGAGTACTA CACCTTCAAG GCAAGCCTCA GTGTTATATC TCGAATATAT
 961 TATATATCAC AACAAACTAA CTAGTCATAC AGTCCGAAGT CACTGCCGTG GACGAAACCG
1021 AACGGACTCG AAACAAGGTC GGCGACGGCC TCAAGATTTA CCTATGGAAA AATCCCTTTC
1081 GCATCCAGGT AGTGCGTCTC TTGACCCCCC TGGTGGACCC TTTCCCCATT CCCAACGTAG
1141 CCAATGCCAC AGCCCGTGTG GCCGACAAGG TTGTTTGGCA GACGTCCCCG AAGACGTTCA
1201 GGAAAAACTT GCATCCGCAG CATAAGATGT TGAAGGATAC AGTTCTTGAT ATTATCAAGC
1261 CGGGGCACGG AGAGTATGTG GGTTGGGGAG AGATGGGAGG CATCGAGTTT ATGAAGGAGC
1321 CAACATTCAT GAATTATTTC AGTAAGCTCT TGAAAGATTT CCTATCTCTT GACGGTCGTT
1381 TTTGCTAAGG AAACTGTAGA CTTTGACAAT ATGCAATATC AGCAGGTCTA TGCACAAGGC
1441 GCTCTTGATA GTCGTGAGCC GTTGTAAGTA ACGTCCTGTG ACATGTCATG ATTACAGTAA
1501 CTGATCGTTC AATAAGGTAT CACTCTGATC CCTTCTATCT CGACGTGAAC TCCAACCCAG
```

FIGURE 15 CONTINUED   19/26

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1561 AGCACAAGAA CATTACGGCA ACCTTTATCG ATAACTACTC TCAGATTGCC ATCGACTTTG
1621 GGAAGACCAA CTCAGGCTAC ATCAAGCTGG GTACCAGGTA TGGCGGTATC GATTGTTACG
1681 GTATCAGCGC GGATACGGTC CCGGAGATTG TGCGACTTTA TACTGGACTT GTTGGGCGTT
1741 CGAAGTTGAA GCCCAGGTAT ATTCTCGGAG CCCACCAAGC TTGTAAGCCC GCCCCCTTTA
1801 CGATGCATTT ATTAGGGGTC CACAGACTAA ACTTGTTCCA AAGGTTATGG ATACCAGCAG
1861 GAAAGTGACT TGCATGCTGT TGTTCAGCAG TACCGTGACA CCAAGTTTCC GCTTGATGGG
1921 TTGCATGTCG ATGTCGACTT TCAGGTAAAT GGCCCAGGTA TCGTTGAAGC TTTGGAGAAT
1981 GCTAATTGTG CTCGTAAAAC TTTAAGGACA ATTTCAGAAC GTTTACCACT AACCCGATTA
2041 CGTTCCCTAA TCCCAAAGAA ATGTTTACCA ATCTAAGGAA CAATGGAATC AAGTGTTCCA
2101 CCAACATCAC CCCTGTTATC AGTATCAGAG ATCGCCCGAA TGGGTACAGT ACCCTCAATG
2161 AGGGATATGA TAAAAAGTAC TTCATCATGG ATGACAGATA TACCGAGGGG ACAAGTGGGG
2221 ACCCGCAAAA TGTTCGATAC TCTTTTTACG GCGGTGGGAA CCCGGTTGAG GTTAACCCTA
2281 ATGATGTTTG GGCTCGGCCA GACTTTGGAG ACAATTAGTA AGTTACTCAA TAGGCTACTT
2341 GAGATATTCT GTAGGTGGCA TTAACACGAC TATAGTGACT TCCCTACGAA CTTCAACTGC
2401 AAAGACTACC CCTATCATGG TGGTGTGAGT TACGGATATG GGAATGGCAC TGTAAGTGAT
2461 AATAAGTCAT AAATACAACG TAATTCATGG AGACTAATCA GTGGTAAATG AATTTTAGCC
2521 AGGTTACTAC CCTGACCTTA ACAGAGAGGA GGTTCGTATC TGGTGGGGAT TGCAGTACGA
2581 GTATCTCTTC AATATGGGAC TAGAGTTTGT ATGGCAAGAT ATGACAACCC CAGCGATCCA
2641 TTCATCATAT GGAGACATGA AAGGGTTGCC CACCCGTCTG CTCGTCACCG CCGACTCAGT
2701 TACCAATGCC TCTGAGAAAA AGCTCGCAAT TGAAAGTTGG GCTCTTTACT CCTACAACCT
2761 CCATAAAGCA ACCTTCCACG GTCTTGGTCG TCTTGAGTCT CGTAAGAACA AACGTAACTT
2821 CATCCTCGGA CGTGGTAGTT ACGCCGGTGC CTATCGTTTT GCTGGTCTCT GGACTGGAGA
2881 TAACGCAAGT ACGTGGGAAT TCTGGAAGAT TTCGGTCTCC CAAGTTCTTT CTCTAGGTCT
2941 CAATGGTGTG TGTATAGCGG GGTCTGATAC GGGTGGTTTT GAGCCCGCAC GTACTGAGAT
3001 TGGGGAGGAG AAATATTGCA GTCCGGAGCT ACTCATCAGG TGGTATACTG GATCATTCCT
3061 TTTGCCATGG CTTAGAAACC ACTACGTCAA GAAGGACAGG AAATGGTTCC AGGTAATATA
```

FIGURE 15 CONTINUED   20/26

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3121 CTCTTTCTGG TCTCTGAGTA TCGAAGACGC TAAGACAATA TAGGAACCAT ACGCGTACCC
3181 CAAGCATCTT GAAACCCATC CAGAGCTCGC AGATCAAGCA TGGCTTTACA AATCTGTTCT
3241 AGAAATTTGC AGATACTGGG TAGAGCTAAG ATATTCCCTC ATCCAGCTCC TTTACGACTG
3301 CATGTTCCAA AACGTGGTCG ATGGTATGCC ACTTGCCAGA TCTATGGTAT GCATTTTATC
3361 CGTCTCCTTT CACGATAATG CACCAGTCTA ACCGAATTTT CTTTTAGCTC TTGACCGATA
3421 CTGAGGATAC GACCTTCTTC AATGAGAGCC AAAAGTTCCT CGATAACCAA TATATGGCTG
3481 GTGACGACAT CCTTGTAGCA CCCATCCTCC ACAGCCGTAA CGAGGTTCCG GGAGAGAACA
3541 GAGATGTCTA TCTCCCTCTA TTCCACACCT GGTACCCCTC AAACTTGAGA CCGTGGGACG
3601 ATCAGGGAGT CGCTTTAGGG AATCCTGTCG AAGGTGGCAG CGTTATCAAC TACACTGCCA
3661 GGATTGTTGC CCCAGAGGAT TATAATCTCT TCCACAACGT GGTGCCGGTC TACATCAGAG
3721 AGGGTAAGCG ATGGAATAAT TTCTTGCAAG TTCCAGATAC AAGTGGTTAC TGACACCTTA
3781 AACCAGGTGC CATCATTCCG CAAATTCAGG TACGCCAGTG GATTGGCGAA GGAGGGCCTA
3841 ATCCCATCAA GTTCAATATC TACCCTGGAA AGGACAAGGT ATATTCTCCA TGACTATCGC
3901 GCATTTATTC TTTCTCTACT CGCACTAACT TCATCTGAAT ATAGGAGTAT GTGACGTACC
3961 TTGATGATGG TGTTAGCCGC GATAGTGCAC CAGATGACCT CCCGCAGTAC CGCGAGGCCT
4021 ATGAGCAAGC GAAGGTCGAA GGCAAAGACG TCCAGAAGCA ACTTGCGGTC ATTCAAGGGA
4081 ATAAGACTAA TGACTTCTCC GCCTCCGGGA TTGATAAGGA GGCAAAGGGT TATCACCGCA
4141 AAGTTTCTAT CAAACAGGTA CATGATTTCA TCTTCCTTTT TTCGCAGTCA CTATTATATC
4201 ATCCTAACAT TGCTTCTCTT ATTTAAAAGG AGTCAAAAGA CAAGACCCGT ACTGTCACCA
4261 TTGAGCCAAA ACACAACGGA TACGACCCCT CTAAGGAAGT TGGTAATTAT TATACCATCA
4321 TTCTTTGGTA CGCACCGGGC TTTGACGGCA GCATCGTCGA TGTGAGCCAG GCGACCGTGA
4381 ACATCGAGGG CGGGGTGGAA TGCGAAATTT TCAAGAACAC CGGCTTGCAT ACGGTTGTAG
4441 TCAACGTGAA AGAGGTGATC GGTACCACAA AGTCCGTCAA GATCACTTGC ACTACCGCTT
4501 AGAGCTCTTT TATGAGGGGT ATATGGGAGT GGCAGCTCAG AAATTTGGGA AGCTTCTGGG
4561 TATTCCTTTT GTTTATTTAC TTATTTATTG AATCGACCAA TACGGGTGGG ATTCTCTCTG
4621 GTTTTTGTGA GGCTATGTTT TACTTGGTCT GAAAATCAAA TTCGTTCTCA
```

FIGURE 16   21/26

```
MC    - MAGFSDPLNFCKAEDYYSVALDWKGPQKIIGVDTTPPKSTKFPKNWHGVN -50
          ::: :::::::::::::::. :  : ::::::  : :::  :: :: :: ::
MV    - MAGLSDPLNFCKAEDYYAAAKGWSGPQKIIRYDQTPPQGTKDPKSWHAVN -50
MC    - LRFDDGTLGVVQFIRPCVWRVRYDPGFKTSDEYGDENTRTIVQDYMSTLS -100
          : :::::. :::::.:::::::::  ::::::::::::::::::.::
MV    - LPFDDGTMCVVQFVRPCVWRVRYDPSVKTSDEYGDENTRTIVQDYMTTLV -100
MC    - NKLDTYRGLTWETKCEDSGDFFTFSSKVTAVEKSERTRNKVGDGLRIHLW -150
          ::  .:::::  .   :::::...:: : :::::. .:::::::::::.: ::
MV    - GNLDIFRGLTWVSTLEDSGEYYTFKSEVTAVDETERTRNKVGDGLKIYLW -150
MC    - KSPFRIQVVRTLTPLKDPYPIPNVAAAEARVSDKVVWQTSPKTFRKNLHP -200
          : ::::::::: :::: ::.:::::: : :::.:::::::::::::::
MV    - KNPFRIQVVRLLTPLVDPFPIPNVANATARVADKVVWQTSPKTFRKNLHP -200
MC    - QHKMLKDTVLDIVKPGHGEYVGWGEMGGIQFMKEPTFMNYFNFDNMQYQQ -250
          :::::::::::::::.::::::::::::::: :::::::::::::::::::
MV    - QHKMLKDTVLDIIKPGHGEYVGWGEMGGIEFMKEPTFMNYFNFDNMQYQQ -250
MC    - VYAQGALDSREPLYHSDPFYLDVNSNPEHKNITATFIDNYSQIAIDFGKT -300
          :::::::::::::::::::::::::::::::::::::::::::::::::::
MV    - VYAQGALDSREPLYHSDPFYLDVNSNPEHKNITATFIDNYSQIAIDFGKT -300
MC    - NSGYIKLGTRYGGIDCYGISADTVPEIVRLYTGLVGRSKLKPRYILGAHQ -350
          ::::::::::::::::::::::::::::::::::::::::::::::::::::
MV    - NSGYIKLGTRYGGIDCYGISADTVPEIVRLYTGLVGRSKLKPRYILGAHQ -350
MC    - ACYGYQQESDLYSVVQQYRDCKFPLDGIHVDVDVQDGFRTFTTNPHTFPN -400
          :::::::::: .:::::: :::::.::::: :: :::::::: ::::
MV    - ACYGYQQESDLHAVVQQYRDTKFPLDGLHVDVDFQDNFRTFTTNPITFPN -400
MC    - PKEMFTNLRNNGIKCSTNITPVISINNREGGYSTLLEGVDKKYFIMDDRY -450
          :::::::::::::::::::::::::  :  ::::: :: ::::::::::
MV    - PKEMFTNLRNNGIKCSTNITPVISIRDRPNGYSTLNEGYDKKYFIMDDRY -450
MC    - TEGTSGNAKDVRYMYYGGGNKVEVDPNDVNGRPDFKDNYDFPANFNSKQY -500
          :::::::   ::: .:::::: ::: :::: :::: :::::::.::: : :
MV    - TEGTSGDPQNVRYSFYGGGNPVEVNPNDVWARPDFGDNYDFPTNFNCKDY -500
MC    - PYHGGVSYGYGNGSAGFYPDLNRKEVRIWWGMQYKYLFDMGLEFVWQDMT -550
          :::::::::::::. :.::::::: :::::::.:: ::: :::::::::::
MV    - PYHGGVSYGYGNGTPGYYPDLNREEVRIWWGLQYEYLFNMGLEFVWQDMT -550
MC    - TPAIHTSYGDMKGLPTRLLVTSDSVTNASEKKLAIETWALYSYNLHKATW -600
          :::::.::::::::::::::::::.:::::::::::::::::::.::::::::.
MV    - TPAIHSSYGDMKGLPTRLLVTADSVTNASEKKLAIESWALYSYNLHKATF -600
```

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FIGURE 16 CONTINUED

```
MC    - HGLSRLESRKNKRNFILGRGSYAGAYRFAGLWTGDNASNWEFWKISVSQV  -650
        :::  :::::::::::::::::::::::::::::::::  :::::::::::

MV    - HGLGRLESRKNKRNFILGRGSYAGAYRFAGLWTGDNASTWEFWKISVSQV  -650

MC    - LSLGLNGVCIAGSDTGGFEPYRDANGVEEKYCSPELLIRWYTGSFLLPWL  -700
        :::::::::::::::::::  :  .    :::::::::::::::::::::::

MV    - LSLGLNGVCIAGSDTGGFEPAR-TEIGEEKYCSPELLIRWYTGSFLLPWL  -699

MC    - RNHYVKKDRKWFQEPYSYPKHLETHPELADQAWLYKSVLEICRYYVELRY  -750
        ::::::::::::::::.:::::::::::::::::::::::::::.:::::

MV    - RNHYVKKDRKWFQEPYAYPKHLETHPELADQAWLYKSVLEICRYWVELRY  -749

MC    - SLIQLLYDCMFQNVVDGMPITRSMLLTDTEDTTFFNESQKFLDNQYMAGD  -800
        :::::::::::::::::::..:::::::::::::::::::::::::::::

MV    - SLIQLLYDCMFQNVVDGMPLARSMLLTDTEDTTFFNESQKFLDNQYMAGD  -799

MC    - DILVAPILHSRKEIPGENRDVYLPLYHTWYPSNLRPWDDQGVALGNPVEG  -850
        :::::::::: :..:::::::::::.:::::::::::::::::::::::::

MV    - DILVAPILHSRNEVPGENRDVYLPLFHTWYPSNLRPWDDQGVALGNPVEG  -849

MC    - GSVINYTARIVAPEDYNLFHSVVPVYVREGAIIPQIEVRQWTGQGGANRI  -900
        :::::::::::::::::::  :::::.:::::::::  :::: : :: : :

MV    - GSVINYTARIVAPEDYNLFHNVVPVYIREGAIIPQIQVRQWIGEGGPNPI  -899

MC    - KFNIYPGKDKEYCTYLDDGVSRDSAPEDLPQYKETHEQSKVEGAEIAKQI  -950
        ::::::::::  :::::::::::::::.:::::.. ::.:::: .. ::.

MV    - KFNIYPGKDKEYVTYLDDGVSRDSAPDDLPQYREAYEQAKVEGKDVQKQL  -949

MC    - G-----KKTGYNISGTDPEAKGYHRKVAVTQTSKDKTRTVTIEPKHNGYD  -995
        :   .  :: : :::::::::.. : :::::::::::::::::::::

MV    - AVIQGNKTNDFSASGIDKEAKGYHRKVSIKQESKDKTRTVTIEPKHNGYD  -999

MC    - PSKEVGDYYTIILWYAPGFDGSIVDVSKTTVNVEGGVEHQVYKNSDLHTV  -1045
        :::::: :::::::::::::::::: .:::.:::::  ....::  ::::

MV    - PSKEVGNYYTIILWYAPGFDGSIVDVSQATVNIEGGVECEIFKNTGLHTV  -1049

MC    - VIDVKEVIGTTKSVKITCTAA  -1066
        :.  ::::::::::::::::..:

MV    - VVNVKEVIGTTKSVKITCTTA  -1070
```

FIGURE 17

```
MAGFSDPLNF  CKAEDYYSVA  LDWKGPQKII  GVDTTPPKST  KFPKNWHGVN  LRFDDGTLGV  VQFIRPCVWR
VRYDPGFKTS  DEYGDENTRT  IVQDYMSTLS  NKLDTYRGLT  WETKCEDSGD  FFTFSSKVTA  VEKSERTRNK
VGDGLRIHLW  KSPFRIQVVR  ILTPLKDPYP  IPNVAAAEAR  VSDKVVWQTS  PKTFRKNLHP  QHKMLKDTVL
DIVKPGHGEY  VGWGEMGGIQ  FMKEPTFMNY  FNFDNMQYQQ  VYAQGALDSR  EPLYHSDPFY  LDVNSNPEHK
NITATFIDNY  SQIAIDFGKT  NSGYIKLGTR  YGGIDCYGIS  ADTVPEIVRL  YTGLVGRSKL  KPRYILGAHQ
ACYGYQQESD  LYSVVQQYRD  CKFPLDGIHV  DVDVQDGFRT  FTTNPHTFPN  PKEMFTNLRN  NGIKCSTNIT
PVISINNREG  GYSTLLEGVD  KKYFIMDDRY  TEGTSGNAKD  VRYMYYGGGN  KVEVDPNDVN  GRPDFKDNYD
FPANFNSKQY  PYHGGVSYGY  GNGSAGFYPD  LNRKEVRIWW  GMQYKYLFDM  GLEFVWQDMT  TPAIHTSYGD
MKGLPTRLLV  TSDSVTNASE  KKLAIETWAL  YSYNLHKATW  HGLSRLESRK  NKRNFILGRG  SYAGAYRFAG
LWTGDNASNW  EFWKISVSQV  LSLGLNGVCI  AGSDTGGFEP  YRDANGVEEK  YCSPELLIRW  YTGSFLLPWL
RNHYVKKDRK  WFQEPYSYPK  HLETHPELAD  QAWLYKSVLE  ICRYYVELRY  SLIQLLYDCM  FQNVVDGMPI
TRSMLLTDTE  DTTFFNESQK  FLDNQYMAGD  DILVAPILHS  RKEIPGENRD  VYLPLYHTWY  PSNLRPWDDQ
GVALGNPVEG  GSVINYTARI  VAPEDYNLFH  SVVPVYVREG  AIIPQIEVRQ  WTGQGGANRI  KFNIYPGKDK
EYCTYLDDGV  SRDSAPEDLP  QYKETHEQSK  VEGAEIAKQI  GKKTGYNISG  TDPEAKGYHR  KVAVTQTSKD
KTRTVTIEPK  HNGYDPSKEV  GDYYIILWY   APGFDGSIVD  VSKTTVNVEG  GVEHQVYKNS  DLHTVVIDVK
EVIGTTKSVK  ITCTAA
```

FIGURE 18   24/26

```
MAGLSDPLNF RKAEDYYAAA KGWSGPQKII RYDQTPPQGT KDPKSWHAVN LPFDDGTMCV VQFVRPCVWR
VRYDPSVKTS DEYGDENTRT IVQDYMTTLV GNLDIFRGLT WVSTLEDSGE YYTFKSEVTA VDETERTRNK
VGDGLKIYLW KNPFRIQVVR LLTPLVDPFP IPNVANATAR VADKVVWQTS PKTFRKNLHP QHKMLKDTVL
DIIKPGHGEY VGWGEMGGIE FMKEPTFMNY FNFDNMQYQQ VYAQGALDSR EPLYHSDPFY LDVNSNPEHK
NITATFIDNY SQIAIDFGKT NSGYIKLGTR YGGIDCYGIS ADTVPEIVRL YTGLVGRSKL KPRYILGAHQ
ACYGYQQESD LHAVVQQYRD TKFPLDGLHV DVDFQDNFRT FTINPITFPN PKEMFTNLRN NGIKCSTNIT
PVISIRDRPN GYSTLNEGYD KKYFIMDDRY TEGTSGDPQN VRYSFYGGGN PVEVNPNDVW ARPDFGDNYD
FPTNFNCKDY PYHGGVSYGY GNGTPGYYPD LNREEVRIWW GLQYEYLFNM GLEFVWQDMT TPAIHSSYGD
MKGLPTRLLV TADSVTNASE KKLAIESWAL YSYNLHKATF HGLGRLESRK NKRNFILGRG SYAGAYRFAG
LWTGDNASTW EFWKISVSQV LSLGLNGVCI AGSDTGGFEP ARTEIGEEKY CSPELLIRWY TGSFLLPWLR
NHYVKKDRKW FQEPYAYPKH LETHPELADQ AWLYKSVLEI CRYWVELRYS LIQLLYDCMF QNVVDGMPLA
RSMLLTDTED TTFFNESQKF LDNQYMAGDD ILVAPILHSR NEVPGENRDV YLPLFHTWYP SNLRPWDDQG
VALGNPVEGG SVINYTARIV APEDYNLFHN VVPVYIREGA IIPQIQVRQW IGEGGPNPIK FNIYPGKDKE
YVTYLDDGVS RDSAPDDLPQ YREAYEQAKV EGKDVQKQLA VIQGNKTNDF SASGIDKEAK GYHRKVSIKQ
ESKDKTRTVT IEPKHNGYDP SKEVGNYYTI ILWYAPGFDG SIVDVSQATV NIEGGVECEI FKNTGLHTVV
VNVKEVIGTT KSVKITCTTA
```
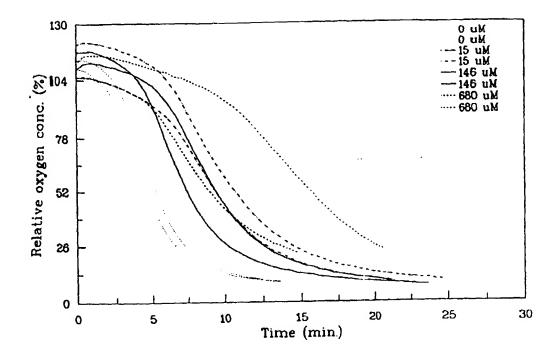
FIG 19

Fig 20